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(54) Title: METHODS AND COMPOSITIONS FOR *IN VIVO* GENE THERAPY

(57) Abstract

    Novel methods and compositions are provided for introducing a gene capable of modulating the genotype and phenotype into two or more tissues following systemic administration. The gene can be introduced into a mammalian host by way of an expression vector either as naked DNA or associated with lipid carriers, particularly cationic lipid carriers. Multiple individual tisssues can be transfected using naked DNA. Using a DNA: lipid carrier complex, multiple tissues and cell types can be transfected. The techniques and compositions find use in the palliation or treatment of any of a variety of genetic-based disorders.

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METHODS AND COMPOSITIONS FOR IN VIVO
GENE THERAPY

INTRODUCTION

5 Technical Field

The present invention relates to methods and compositions for systemic introduction of exogenous genetic material into mammalian, particularly human, cells *in vivo*.

10 Background

An ever-expanding array of genes for which abnormal expression is associated with life-threatening human diseases is being cloned and identified. The ability to express such cloned genes in humans will

15 ultimately permit the prevention and/or cure of many important human diseases, diseases which now either are treated poorly or are untreatable by currently available therapies. As an example, *in vivo* expression of cholesterol-regulating genes, genes which selectively

20 block the replication of HIV, or of tumor-suppressing genes in human patients should dramatically improve treatment of heart disease, HIV, and cancer, respectively. However, currently available gene delivery strategies have been unable to produce either a high

25 level of or generalized transgene expression *in vivo* in a wide variety of tissues after systemic administration to a mammalian host. This inability has precluded the development of effective gene therapy for most human diseases.

30 Approaches to gene therapy include both different goals and different means of achieving those goals. The goals generally include gene replacement, gene correction and gene augmentation. In gene replacement, a mutant

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gene sequence is specifically removed from the genome and
replaced with a normal, functional gene. In gene
correction, a mutant gene sequence is corrected without
any additional changes in the target genome. In gene
5 augmentation, the expression of mutant genes in defective
cells is modified by introducing foreign normal genetic
sequences.

     The means to reach the above goals used by others,
have included "ex vivo" transfection of a target cell
10 followed by introduction of the transformed cells into a
suitable organ in the host mammal. Ex vivo techniques
include transfection of cells in vitro with either naked
DNA or DNA liposome conjugates, followed by introduction
into a host organ ("ex vivo" gene therapy). The criteria
15 for a suitable target organ or tissue include that the
target organ or tissue is easily accessible, that it can
be manipulated in vitro, that it is susceptible to
genetic modification methods and ideally, it should
contain either non-replicating cells or cycling stem
20 cells to perpetuate a genetic correction. Further, it
should be possible to reimplant the genetically modified
cells into the organism in a functional and stable form.
Exemplary of a target organ which meets these criteria is
the mammalian bone marrow. A further criterion for
25 ex vivo gene therapy, if for example a retroviral vector
is used, is that the cells be pre-mitotic; post-mitotic
cells are refractory to infection with retroviral
vectors. Although this has not been reported, in some
instances it may be possible to transfect cells from
30 other than the target organ or tissue using ex vivo gene
therapy if the corrective gene product can be secreted
and exert the desired effect on/in the target cell
following circulation in blood or other body fluids.
     There are several drawbacks to ex vivo therapy;
35 for example, if only differentiated, replicating cells

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are infected, the newly introduced gene function will be
lost as those cells mature and die. *Ex vivo* approaches
also can be used to transfect only a limited number of
cells and cannot be used to transfect cells which are not
5 removed first from the body. The above methods generally
involve integration of new genetic material into the cell
genome and thus constitute permanent changes.

Liposomes have been used effectively, particularly
to introduce drugs, radiotherapeutic agents, enzymes,
10 viruses, transcription factors and other cellular
effectors into a variety of cultured cell lines and
animals. The agent to be introduced is typically
entrapped within the liposome, or lipid vesicle, or the
agent may be bound to the outside of the vesicle.
15 Successful clinical trials examining the effectiveness of
liposome-mediated drug delivery have been completed.
Several strategies have been devised to increase the
effectiveness of liposome-mediated drug delivery by
targeting liposomes to specific tissues and specific cell
20 types. However, while the basic methodology for using
liposome-mediated vectors is well developed, the
technique has not been perfected for liposome-based
transfection vectors for *in vivo* gene therapy.

In vivo expression of transgenes as reported by
25 others has been restricted to injection of transgenes
directly into a specific tissue, such as direct
intratracheal, intramuscular or intraarterial injection
of naked DNA or of DNA-cationic liposome complexes, or to
ex vivo transfection of host cells, with subsequent
30 reinfusion. The expression is low and generally has been
limited to one tissue, typically the tissue that was
injected (for example muscle); liver or lung where iv
injection has been used; or lung where intratracheal
injection has been used, and less than 1% of all cells
35 within these tissues were transfected. In some cases,

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transfection of cells has been obtained in tissues
afferent to the site of intravenous administration.

Currently available gene delivery strategies
consistently have failed to produce a high level and/or
5 generalized transgene expression *in vivo*. It therefore
would be of interest to develop compositions and delivery
methods for *in vivo* gene therapy that provide for a high
level of transcription of the transgene and/or expression
in a variety of cell and tissue types for the *in vivo*
10 treatment, prevention, or palliation of numerous human
diseases. Also of interest, is the use of gene
modulation as an alternate means of gene therapy. In
gene modulation, expression of an already expressed gene
is increased by introducing exogenous normal genetic
15 sequences and decreased by introducing antisense genes or
gene fragments, or by introducing vectors that can
produce ribozymes that can cleave specific mRNAs. Gene
modulation can also be achieved by the introduction of
exogenous normal genetic sequences that code for proteins
20 that modulate the extent of gene expression, or affect
the processing, assembly or secretion of gene products.

Relevant Literature

A large number of publications relate to *in vivo*
and *ex vivo* transfection of mammals. In some cases, only
25 transcription of a transgene has been achieved, in
others, the data appear to show only a low level of
expression and/or expression in a limited number of
tissues or cell types. The following are examples of the
publications in this area.
30 A variety of approaches for introducing functional
new genetic material into cells, both *in vitro* and *in
vivo* have been attempted (Friedmann, (1989) *Science*,
244:1275-1281). These approaches include integration of
the gene to be expressed into modified retroviruses

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(Friedmann, (1989) *supra*; Rosenberg, (1991) *Cancer
Research*, <u>51</u>(18), <u>suppl.</u>: 5074S-5079S); integration into
non-retrovirus vectors (Rosenfeld, *et al.*, (1992) *Cell*,
<u>68</u>:143-155; Rosenfeld, *et al.*, (1991) *Science*, <u>252</u>:431-
5 434); or delivery of a transgene linked to a heterologous
promoter-enhancer element via liposomes (Friedmann,
(1989), *supra*; Brigham, *et al.*, (1989) *Am. J. Med. Sci.*,
<u>298</u>:278-281; Nabel, *et al.*, (1990) *Science*, <u>249</u>:1285-
1288; Hazinski, *et al.*, (1991) *Am. J. Resp. Cell Molec.*
10 *Biol.*, <u>4</u>:206-209; and Wang and Huang, (1987) *Proc. Natl.
Acad. Sci. (USA)*, <u>84</u>:7851-7855); coupled to ligand-
specific, cation-based transport systems (Wu and Wu,
(1988) *J. Biol. Chem.*, <u>263</u>:14621-14624) or the use of
naked DNA expression vectors (Nabel *et al.*, (1990),
15 *supra*); Wolff *et al.*, (1990) *Science*, <u>247</u>:1465-1468).
Direct injection of transgenes into tissue produces only
localized expression (Rosenfeld, (1992) *supra*); Rosenfeld
et al., (1991) *supra*; Brigham *et al.*, (1989) *supra*;
Nabel, (1990) *supra*; and Hazinski *et al.*, (1991) *supra*).
20 The Brigham *et al.* group (*Am. J. Med. Sci.*, (1989)
<u>298</u>:278-281 and *Clinical Research*, (1991) 39 (abstract))
have reported *in vivo* transfection only of lungs of mice
following either intravenous or intratracheal
administration of a DNA liposome complex. An example of
25 a review article of human gene therapy procedures is:
Anderson, (1992) *Science* <u>256</u>:808-813.

        PCT/US90/01515 (Felgner *et al.*) is directed to
methods for delivering a gene coding for a pharmaceutical
or immunogenic polypeptide to the interior of a cell of a
30 vertebrate *in vivo*. Expression of the transgenes is
limited to the tissue of injection. PCT/US90/05993
(Brigham) is directed to a method for obtaining
expression of a transgene in mammalian lung cells
following either iv or intratracheal injection of an
35 expression construct. PCT 89/02469 and PCT 90/06997 are

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directed to *ex vivo* gene therapy, which is limited to
expressing a transgene in cells that can be taken out of
the body such as lymphocytes. PCT 89/12109 is likewise
directed to *ex vivo* gene therapy. PCT 90/12878 is

5 directed to an enhancer which provides a high level of
expression both in transformed cell lines and in
transgenic mice using *ex vivo* transfection.
PCT/US92/08806 is directed to particle-mediated
transformation of mammalian unattached cells. EP

10 application 91301819.8 is directed to the use of
recombinant techniques to produce cystic fibrosis
transmembrane conductance regulator (CFTR).

SUMMARY OF THE INVENTION

Methods and compositions are provided for

15 introduction of a transgene into a plurality of mammalian
tissues *in vivo*. The method includes the step of
incorporating a transfection cassette comprising a
nucleotide sequence of interest into a largely non-
integrating plasmid and introducing the plasmid into a

20 mammalian host, other than by directly introducing it
into a specific tissue. The transfection cassette
comprises as operably joined components, a
transcriptional and initiation regulatory region, a
nucleic acid sequence of interest, and a transcriptional

25 termination regulatory region, wherein said regulatory
regions are functional in the cells of a mammalian host,
and wherein said nucleic acid sequence of interest is
free of introns. Optionally, the transcriptional
cassette is complexed with a cationic lipid carrier. The

30 method finds use to modulate the phenotype of mammalian
cells.

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DESCRIPTION OF THE DRAWINGS

Figure 1A shows photomicrographs of sections of mouse lung from a mouse injected with a transfection cassette, or Figure 1B a control mouse. The

5 photomicrograph in 1A shows a section of mouse lung 48 hours following iv injection of pZN27:DDAB:Cholesterol expression vector-cationic lipid carrier complexes. The plasmid contained sequences for the expression of CAT (chloramphenicol acyl transferase). The lipid carrier

10 composition was 1 to 1 molar DDAB:Cholesterol. The carrier: plasmid ratio was 5 nmoles cationic lipid to 1 μg DNA. A dose of 100 μg DNA was injected per mouse. This field shows alveoli and alveolar lining cells, the majority (50-70%) of which stain red positively for the

15 presence of CAT protein when probed with anti-CAT antibody and visualized using alkaline phosphatase (indicated by arrows). The treated animals' lungs stain uniformly with diffuse involvement of alveolar and vascular endothelial cells. Airway epithelial staining

20 is also seen indicating airway cells are also transfected. The CAT protein normally is not present in mammalian cells and therefore the presence of CAT protein in these cells indicates that they have been transfected *in vivo*. The photomicrograph in 1B shows a section of

25 mouse lung from a control animal treated with iv-injected lipid carrier only, and probed with anti-CAT antibody. Cells do not show significant staining, although low-level background staining is detectable in some alveolar macrophages (indicated by arrows), which possess

30 endogenous alkaline phosphatase activity.

Figure 2A shows a phase contrast micrograph of mouse T lymphocytes isolated 48 hours after intraperitoneal (ip) injection of DNA-cationic lipid carrier complexes. Lipid carriers were DDAB:DOPE, 1 to

35 1 molar. DNA-cationic lipid carrier complexes were 1 mg

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pCIS-CAT with 1 μmole cationic lipid. 1 mg DNA was
injected *ip*. Cells were incubated with anti CAT mouse
monoclonal antibody, followed by Texas red-conjugated
goat anti mouse IgG. Figure 2B shows a fluorescence
5   micrograph of the same field showing that essentially all
T lymphocytes present removed from the treated animal
were stained red by immunofluorescence demonstrating
expression of the transfected CAT gene following ip
injection of DNA/cationic lipid carrier complexes.
10 Figure 2C shows a phase contrast micrograph of T
lymphocytes isolated from an uninjected control mouse.
Figure 2D shows a fluorescence micrograph of the same
field, showing that there is no CAT gene expression in
these lymphocytes from control animals.
15   Figure 3A shows hematopoietic bone marrow-derived
cells from a mouse treated with a DNA:lipid carrier
complex DDAB:Cholesterol:pZN51 intravenously 48 hours
earlier. Cells were stained for CAT protein as in Figure
1. The photomicrograph shows that approximately 70
20 percent of the bone marrow-derived cells, including
primitive or blast cells are stained red (blast cell
indicated by arrow) and are transfected *in vivo*. Lipid
carriers were 1:1 DDAB:Cholesterol. DNA:cationic lipid
ratio was 1 μg of DNA to 5 nmoles cationic lipid. 100 μg
25 of DNA was injected iv into each mouse. Figure 3B shows
isolated bone marrow cells from an untreated control
mouse. The photomicrograph shows no red staining
indicating no CAT gene expression in untreated animals.
        Figures 4A-4B show transfection of human T
30 lymphocytes (CD4$^+$) by CAT expression plasmid-DNA
complexes in culture. The lipid carrier composition was
1:1 molar ratio DDAB:DOPE small uni-lamellar vesicles
(SUV). 25 μg of pZN27 complexed with 50 nmoles cationic
lipid was added to 10 million cells in culture. Cells
35 were incubated with anti CAT mouse monoclonal antibody,

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followed by Texas red-conjugated goat anti mouse IgG. In
Figure 4A is shown a phase contrast micrograph of freshly
isolated human CD4$^+$ T lymphocytes 48 hours after
transfection. Figure 4B shows a fluorescence micrograph

5 of the same field which demonstrates that essentially
100% of the T lymphocytes stain positively for CAT
protein, i.e., they have been transfected.

Figure 5 shows construction of plasmid pZN20.

Figure 6A shows a restriction map of the immediate

10 early enhancer and promoter region of HCMV (Towne) and
HCMV(AD169) is shown in Figure 6C. Figure 6B shows a
sequence comparison of the two HCMV promoters. The
position of the NcoI site is indicated by an asterisk.

Figure 7 (panels a-f) shows electron micrographs

15 which demonstrates that cationic lipid carrier: DNA
complexes (DOTMA:DOPE:pRSV-CAT) are internalized by CV-1
(African Green Monkey Kidney) cells via classical
receptor-mediated endocytosis following binding to cell
surface receptors. For construction of pRSVCAT see

20 Gorman et al., (1982) Proc. Nat. Acad. Sci. USA 79:6777-
6781. Lipid carriers were 1:1 DOTMA:DOPE. 20 µg DNA
were complexed with 20 nmoles cationic lipid, and added
to 2×10^6 cells. The arrows in panel (a) show particles
binding to clathrin-coated pits; panel (b), the particles

25 that have been ingested and are present in endosomes.

Figures 8A-8C show photomicrographs of a
histochemical analysis of lungs from B-16 melanoma-
bearing animals which received an intravenous injection
of CMV-CAT cationic lipid carrier:DNA complexes. Lipid

30 carriers were DOTAP:Cholesterol, 1 to 1 molar ratio.
Cationic lipid: DNA ratio was 6 nanomoles: 1 µg DNA. The
plasmid used was pZN20 (see Figure 5). 100 µg DNA was
injected per mouse. The immunohistochemical analysis for
CAT protein revealed intense red staining of many focal

35 parenchymal tumors (Figure 8A indicated by arrows) and

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tumor emboli within blood vessels (Figure 8B indicated by
arrows), indicating that large numbers of B16 melanoma
tumor cells in the lung, as well as blood-borne
metastases, are transfected after iv injection. Figure
5   8C shows that in tissue from B16 melanoma-bearing mice
which did not receive an injection of DNA-lipid carrier
complexes, no CAT protein is present in the surrounding
normal lung or in any of the lung tumor cells.

        Figure 9 shows the construction of plasmid pZN27,
10 encoding CAT.

        Figure 10A and Figure 10B show the construction of
plasmid pZN46, encoding human IL-2.

        Figure 11 shows the construction of plasmid pZN32,
encoding human CFTR.

15   Figures 12A-12E shows photomicrographs of
immunohistochemically stained (for CFTR protein) frozen
sections of lung tissue from mice treated by intravenous
administration of either pZN32:cationic lipid carrier
complexes or lipid carrier alone (control mice). Figures
20 12A, 12C, and 12E are lung sections from mice treated
with DNA lipid carrier complexes at 50X, 100X, and 250X
magnification, respectively. Figures 12B and 12D are
lung sections from control mice at 50X and 100X
magnification show that there is no detectable CFTR
25 expression in lungs of control mice. Lipid carriers were
1 to 1 molar DDAB:Cholesterol (SUV). Lipid carrier-DNA
complexes were 5 nanomoles cationic lipid to 1 μg DNA.
100 μg DNA was injected per mouse. Animals were
sacrificed 24 hrs after injection. The figures show that
30 >70% of both airways and airway lining cells stain
intensely red, indicating that they are expressing the
CFTR gene.

        Figure 13 shows the construction of plasmid pZN51,
encoding CAT.

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Figure 14 shows the construction of plasmids pZN60, pZN61, pZN62 and pZN63, all encoding CAT. Figures 14A and 14B show the construction of intermediate plasmids pZN52, pZN54, pZN56 and pZN58. Figure 14C shows
5 the construction of the final plasmids, pZN60 through pZN63, from the intermediates.

Figure 15 shows an autoradiograph of the thin layer chromatograph of the a CAT assay for six different plasmids injected intravenously in mice. Lanes 1-12 show
10 the CAT activity in lung tissue; Lanes 13-24 show the CAT activity in liver tissue. Lanes 1, 2, 13, 14-pZN51; lanes 3, 4, 15, 16-pZN60; lanes 5, 6, 17, 18-pZN61; lanes 7, 8, 19, 20-pZN62; lanes 9, 10, 21, 22-pZN63; lanes 11, 12, 23, 24-pZN27. Lipid carriers were DDAB:Cholesterol
15 (1:1). Lipid carriers-DNA complexes were 5 nmoles cationic lipid to 1μg DNA. 100μg DNA was injected per mouse. Animals were sacrificed after 48 hrs. Each lane represents a single mouse. Chromatograph runs from bottom to top of Figure as shown. As shown by the
20 brackets in Figure 15, unreacted ^{14}C chloramphenicol only migrates a short distance from the origin of the TLC plate. The ^{14}C Acetylated chloramphenicol species migrate which result from CAT enzymatic activity further up the plate as shown by the brackets.
25   Figures 16A-16E show photomicrographs of frozen lung section that have been immunohistochemically stained for CAT protein. Mice were injected iv by tail vein with pZN27 (16A) which shows positive red staining for CAT is endothelial, alveolar and airway cells within the lung.
30 In contrast, liposome complexes of the CAT gene linked to the natural CFTR promoter (pBE3.8CAT) showed CAT expression primarily in airway epithelial cells (16B). A lung section from an uninjected control animal did not show red staining, indicating no CAT expression in
35 untransfected cells (16C). Figure 16D shows a high

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magnification photomicrograph of alveoli from a pZN27
treated mouse, and shows a high level of positive red
staining for CAT gene product in both alveolar and
epithelial cells. Figure 16E shows a high magnification

5 photomicrograph of alveoli from a pBE3.8CAT injected
mouse, and shows no significant CAT gene expression in
either alveolar or endothelial cells. One hundred μg of
plasmid was injected iv complexed with DDAB:Cholesterol
as SUVs. Animals were sacrificed after 24 hrs.

10   Figures 16F-16K show autoradiographs of the thin
layer chromatographs of CAT activity in heart (16F),
lymph nodes (16G) spleen (16H), kidney (16I), lung (16J),
and liver (16K) in tissue from uninjected mice (lanes 1-
3), mice injected IV with pBE3.8CAT (Chou, et al., (1991)

15 J. Biol. Chem., 266:24471-24476 (lanes 4-6), or mice
injected with pCIS-CAT (lanes 7-9). Lipid carriers were
DDAB:Cholesterol, 1:1 SUV (1 μg DNA to 5 nmoles cationic
lipid; 100 μg injected per mouse.

        Figure 17 shows the construction of plasmid pZN84,

20 encoding murine GM-CSF.

        Figure 18 shows the construction of pZN102,
encoding human CFTR.

        Figure 19A shows an autoradiograph of a TLC plate
showing CAT activity in animals after injection of pZN27

25 without any lipid carrier. One milligram was injected iv
twice over a 4-hour period. Mice were sacrificed 24 hrs.
later. The various tissues examined are indicated under
each lane.

        Figure 19B shows an autoradiograph of a TLC plate

30 showing CAT activity in an animal injected with pZN27
complexed with a lipid carrier. Lipid carrier was
DDAB:Cholesterol 1 to 1 molar, 5 μg of plasmid complexed
to 1 nmole of lipid. 100 μg was injected iv, and animals
were sacrificed 24 hrs later. The various tissues

35 examined are indicated under each lane.

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Figure 20 shows an autoradiograph of a TLC plate
of injection into the right (R) ventricle of pCIS-CAT
complexed to DOTMA:DOPE (1:1) lipid carriers in the
indicated ratios of plasmid:lipid. Animals were
5   sacrificed 48 hrs. after the stereotactic injection of
2.5 μg. Each pair of lanes represents a single animal.
The left (L) ventricle was not injected and therefore
transfection of the left ventricles demonstrates that the
entire brain is being transfected.

10   Figure 21 shows human lung cancer cell line
transfections. An autoradiograph of a TLC plate shows
CAT activity in extracts from three different human lung
cancer cell lines transfected using various cationic
liposomes. The lung cancer cell lines NCI-H69, NCI-H82,
15 and NCI-H520 were plated at 2×10^6 cells/plate and
transfected in suspension. Individual plates of cells
were transfected with 5 μg of RSV-CAT (RC) alone or 5 μg
of RSV-CAT complexed to a total of 10 or 20 nmole of pure
DOTMA (D), pure L-PE (L), or L-PE/CEBA (L/CA) liposomes.
20 The cells were harvested 48 h after transfection and
assayed for CAT activity.

Figure 22 shows serum murine GM-CSF levels in a
single goat (A) injected iv with 1 mg of GM-CSF plasmid
(pZN84) complexed with DDAB:Cholesterol liposome (1 μg
25 plasmid to 1 nmole lipid). Serum samples were collected
at 0, 12, 24, 48, 72, 168, and 840 hrs. after injection.
Murine GM-CSF levels were measured by ELISA using a
commercial kit (Endogen).

Figure 23 show the serum murine GM-CSF levels in
30 two additional goats (B and C) injected with pZN84 as per
goat A.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, nucleic
acid constructs together with methods of preparation and

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use are provided which provide for *in vivo* change of
and/or modulation of phenotype of cells in a plurality of
tissues of a mammalian host, following introduction of
the constructs into the host at a dose sufficient to
5 cause transfection of tissues and cells contacted by the
nucleic acid. The components of the transfection vector
generally will include as operably linked components in
the direction of transcription, a transcriptional
initiation region, a DNA sequence of interest and a
10 transcriptional termination region, wherein the
transcriptional regulatory regions are functional in the
host mammal's cells that are targeted for transfection.
Optionally, an intron may be included in the construct,
preferably 5' to the coding sequence. Generally, the
15 construct does not become integrated into the host cell
genome and is introduced into the host as part of a non-
integrating plasmid where it is maintained primarily in
an extrachromosomal or episomal form. The constructs can
be either naked nucleic acid or nucleic acid associated
20 with a lipid carrier. By a sufficient dose is meant that
which will result in a desired effect, for example
prevention, palliation, and/or cure of an animal or human
disease, or modulation of endogenous levels of an agent
of interest ("*in vivo*" gene therapy). The modulation may
25 be generalized, i.e. obtained in a multiplicity of cell
types or tissues, or the modulation may be selective, for
example inducible and/or in only selected cell or tissue
types.

Transfection of multiple tissues and cells other
30 than those solely at or afferent to the site of
introduction of the nucleic acid constructs into the host
is obtained and expression is at a high level and in a
large number of cells and cell types. The tissues which
can be transformed include the lungs, heart, liver, bone
35 marrow, spleen, lymph nodes, kidneys, thymus, skeletal

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muscle, ovary, uterus, stomach, small intestine, colon,
pancreas, and brain in normal animals, as well as
metastatic tumors and intravascular tumor emboli in
tumor-bearing mammals. Particular cells which are
5 transfected include macrophages, alveolar type I and type
II cells, hepatocytes, airway epithelial cells, vascular
endothelial cells, cardiac myocytes, myeloblasts,
erythroblasts, B-lymphocytes and T-lymphocytes. The
route of administration typically is into a circulating
10 bodily fluid such as blood or cerebrospinal fluid, but
other routes of administration also can be used. The
constructs can be either naked nucleic acid or nucleic
acid associated with a lipid carrier. Optionally, the
lipid carrier molecule and/or construct may provide for
15 targeting and/or expression in a particular cell type or
types.

The nucleic acid constructs can be prepared from
nucleic acid sequences which are synthetic, naturally
derived or a combination thereof. The sequences can be
20 obtained from a donor host homologous with an intended
recipient host, or depending upon the nature of the
nucleic acid sequence, it may be desirable to synthesize
the sequence with host preferred codons. The host
preferred codons may be determined from the codons of
25 highest frequency in the proteins expressed in the
largest amount in the particular host of interest. Where
cloning is done in a bacterial host system, it may be
beneficial to change codon choice to promote stability in
the bacterial host system. Where the intended use is to
30 treat disease resulting from an infectious organism, an
appropriate source of sequence may be viral nucleic acid,
either RNA or DNA.

Genes are high molecular weight, polyanionic
molecules, for which carrier-mediated delivery is usually
35 but not always required for DNA transfection of cells

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either in culture or *in vivo*. Cationic lipid carriers
such as liposomes can be used to deliver transcriptional
regulatory proteins. Further, liposomes themselves
(unlike viral vectors) appear to be non-immunogenic
5 *in vivo*. Liposome formulations, including those
containing a cationic lipid, have been shown to be safe
and well tolerated in human patients (Treat *et al*.,
(1990) *J. Natl. Cancer Instit*. 82:1706-1710). Although a
wide variety of transfection techniques can produce high
10 level expression of transgenes in cultured cells, only a
few such methods, including those using liposomes, are
compatible with *in vivo* gene delivery. Previous attempts
at using either cationic liposomes or naked DNA to obtain
in vivo transfection have resulted in transfection of a
15 single tissue and/or transfection only of the tissue of
introduction of exogenous DNA. This pattern of
transfection suggests that rather than the DNA and/or
liposomes being taken up by normal cellular mechanisms,
cells in the tissue of introduction are damaged by the
20 introduction method and are then able to take up some of
the exogenous DNA which has been introduced.

Applicants surprisingly discovered changes which
provide for both transfection of a wide variety of
tissues and cell types and a high-level of transgene
25 expression after systemic administration into a mammalian
host. These changes include the use of DNA:cationic
lipid carrier complexes, wherein the ratio of DNA to
cationic lipid of the lipid carrier can significantly
affect obtention of *in vivo* expression; use of a higher
30 dose of complexes than has been used previously; use of
an appropriate promoter element, for example one which is
both strong and constitutively active, such as the HCMV-
IE1 element where it is desired to provide enhanced
expression in a wide variety of cell types *in vivo*; and
35 placement of greater than 100 bp of an intron 5' to the

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coding region of the transgene or removal of the intron altogether to facilitate production of a desired gene product. Additionally, it has surprisingly been discovered that a number of tissues, including the lung,

5   liver, spleen, kidney, lymph nodes and heart can be transformed following direct administration of high doses of naked DNA into a circulating bodily fluid, such as blood or cerebral spinal fluid. Alternatively, selective expression can be obtained in specific cell and tissue

10   types and at a desired time (i.e. expression is inducible) by alterations in the components, particularly promoters and/or enhancers of the constructs used, or by the use of targeting moieties on the liposomes.

Most gene therapy strategies have relied on

15   transgene insertion into retroviral or DNA virus vectors. Potential disadvantages of retroviruses, as compared to the use of naked DNA or the use of cationic lipid carriers, include the limited ability of retroviruses to mediate *in vivo* (as opposed to *ex vivo*) transgene

20   expression; the inability of retrovirus vectors to transfect non-dividing cells; possible recombination events in replication-defective retrovirus vectors, resulting in infectious retroviruses; possible activation of oncogenes or inhibition of tumor suppressor genes due

25   to the random insertion of the transgene into host cell genomic DNA; size limitations: less than 15 kb of DNA typically can be packaged in a retrovirus vector; and potential immunogenicity, leading to a host immune response against the vector. In addition, all *ex vivo*

30   approaches require that the cells to be transfected be removed from the body and that they be maintained in culture for a period of time. While in culture, the cells may undergo deleterious or potentially dangerous phenotypic and/or genotypic changes. Adenoviral and

35   other DNA viral vectors share several of the above

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potential limitations. Thus, the subject invention which
does not use adenoviral or other DNA viral vectors has
several advantages over existing techniques.
Additionally, the subject invention offers ease of
5 administration and results not achievable by other means.
        The constructs for use in the invention include
several forms, depending upon the intended use of the
construct. Thus, the constructs include vectors,
transcriptional cassettes, expression cassettes and
10 plasmids. The transcriptional and translational
initiation region (also sometimes referred to as a
"promoter,"), preferably comprises a transcriptional
initiation regulatory region and a translational
initiation regulatory region of untranslated 5'
15 sequences, "ribosome binding sites," responsible for
binding mRNA to ribosomes and for translational
initiation. It is preferred that all of the
transcriptional and translational functional elements of
the initiation control region are derived from or
20 obtainable from the same gene. In some embodiments, the
promoter will be modified by the addition of sequences,
such as enhancers, or deletions of nonessential and/or
undesired sequences. By "obtainable" is intended a
promoter having a DNA sequence sufficiently similar to
25 that of a native promoter to provide for the desired
specificity of transcription of a DNA sequence of
interest. It includes natural and synthetic sequences as
well as sequences which may be a combination of synthetic
and natural sequences.
30   For the transcriptional initiation region, or
promoter element, any region may be used with the proviso
that it provides the desired level of transcription of
the DNA sequence of interest. The transcriptional
initiation region may be native to or homologous to the
35 host cell, and/or to the DNA sequence to be transcribed,

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or nonindigenous to the host cell or foreign or
heterologous to the DNA sequence to be transcribed. By
nonindigenous to the host cell is intended that the
transcriptional initiation region is not normally found
5   in the host into which the construct comprising the
transcriptional initiation region is to be inserted. By
foreign to the DNA sequence is intended a transcriptional
initiation region that is not normally associated with
the DNA sequence of interest. Efficient promoter
10 elements for transcription initiation include the SV40
(simian virus 40) early promoter, the RSV (Rous sarcoma
virus) promoter, the Adenovirus major late promoter, and
the human CMV (cytomegalovirus) immediate early 1
promoter.
15   Inducible promoters also find use with the subject
invention where it is desired to control the timing of
transcription or the tissue or cell type in which the
nucleic acid of interest is transcribed. Examples of
inducible promoters include those obtainable from a
20 β-interferon gene, a heat shock gene, a metallothionein
gene or those obtainable from steroid hormone-responsive
genes, including insect genes such as that encoding the
ecdysone receptor. Such inducible promoters can be used
to regulate transcription of the transgene by the use of
25 external stimuli such as interferon or glucocorticoids.
Since the arrangement of eukaryotic promoter elements is
highly flexible, combinations of constitutive and
inducible elements also can be used. Tandem arrays of
two or more inducible promoter elements may increase the
30 level of induction above baseline levels of transcription
which can be achieved when compared to the level of
induction above baseline which can be achieved with a
single inducible element.
        Generally, the transcriptional regulatory sequence
35 comprises DNA up to about 1.5 kb 5' of the

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transcriptional start of a gene, but can be significantly
smaller. As necessary, this regulatory sequence may be
modified at the position corresponding to the first codon
of the desired protein by site-directed mutagenesis
5 (Kunkel, (1985) *Proc. Natl. Acad. Sci. (USA)*, 82:488-492)
or by introduction of a convenient linker oligonucleotide
by ligation, if a suitable restriction site is found near
the N-terminal codon. In the ideal embodiment, a coding
sequence with a compatible restriction site may be
10 ligated at the position corresponding to codon #1 of the
gene. This substitution may be inserted in such a way
that it completely replaces the native coding sequence so
that the substituted sequence is flanked at its 3' end by
the gene terminator and polyadenylation signal.
15   Transcriptional enhancer elements optionally may
be included in the transcription or expression cassette.
By "transcriptional enhancer elements" is intended DNA
sequences which are primary regulators of transcriptional
activity and which can act to increase transcription from
20 a promoter element, and generally do not have to be in
the 5' orientation with respect to the promoter in order
to enhance transcriptional activity. The combination of
promoter and enhancer element(s) used in a particular
cassette can be selected by one skilled in the art to
25 maximize specific effects. Different enhancer elements
can be used to produce a desired level of transgene
transcription and/or expression in a wide variety of
tissue and cell types. For example, the human CMV
immediate early promoter-enhancer element can be used to
30 produce high level transgene transcription and expression
in many different tissues *in vivo*.
        Examples of other enhancer elements which confer a
high level of transcription on linked genes in a number
of different cell types from many species include
35 enhancers from SV40 and RSV-LTR. The SV40 and RSV-LTR

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are essentially constitutive. They can be combined with
other enhancers which have specific effects, or the
specific enhancers may be used alone. Thus, where
specific control of transcription is desired, efficient
5 enhancer elements that are active only in a tissue-,
developmental-, or cell-specific fashion include
immunoglobulin, interleukin-2 (IL-2) and β-globin
enhancers are of interest. Tissue-, developmental-, or
cell-specific enhancers can be used to obtain transgene
10 transcription and/or expression in particular cell types,
such as B-lymphocytes and T-lymphocytes, as well as
myeloid, or erythroid progenitor cells. Alternatively, a
tissue-specific promoter such as that derived from the
human cystic fibrosis transmembrane conductance regulator
15 (CFTR) gene can be fused to a very active, heterologous
enhancer element, such as the SV40 enhancer, in order to
confer both a high level of transcription and tissue-
specific transgene transcription. In addition, the use
of tissue-specific promoters, such as LCK, allow
20 targeting of transgene transcription to T lymphocytes.
Tissue specific transcription of the transgene can be
important, particularly in cases where the results of
transcription of the transgene in tissues other than the
target tissue would be deleterious.
25   Tandem repeats of two or more enhancer elements or
combinations of enhancer elements can significantly
increase transgene expression when compared to the use of
a single copy of an enhancer element; hence enhancer
elements find use in the cassettes. The use of two
30 different enhancer elements, which can be either from the
same or from different sources flanking or within a
single promoter can in some cases produce transgene
expression in each tissue in which each individual
enhancer acting alone would have an effect, thereby
35 increasing the number of tissues in which transcription

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is obtained. In other cases, the presence of two
different enhancer elements results in silencing of the
enhancer effects. Evaluation of particular combinations
of enhancer elements for a particular desired effect or
5 tissue of expression is within the level of skill in the
art.

Although generally it is not necessary to include
an intron in the transcription and/or expression
cassette, an intron optionally can be included which
10 comprises a 5' splice site (donor site) and a 3' splice
site (acceptor site) separated by a sufficient
intervening sequence to produce high level extended
in vivo transcription and/or expression of a transgene.
Generally, an intervening sequence of about 100bp
15 produces the desired expression pattern and/or level, but
the size of the sequence can be varied as needed to
achieve a desired result. The optional intron placed 5'
to the coding sequence results in high level extended in
vivo expression of a transgene but generally is not
20 necessary to obtain expression. Optimally, the 5' intron
specifically lacks cryptic splice sites which result in
aberrantly spliced mRNA sequences.

If used, the intron splice donor and splice
acceptor sites, arranged from 5' to 3' respectively, are
25 placed between the transcription initiation site and the
translational start codon. Alternatively, the
intervening sequence can be placed 3' to the
translational stop codon and the transcriptional
terminator or inside the coding region. The intron can
30 be a hybrid intron with an intervening sequence or an
intron taken from a genomic coding sequence. An intron
3' to the coding region, particularly one of less than
100 bp, or any intron which contains cryptic splice sites
can under certain condition substantially reduce the
35 level of transgene expression produced in vivo. However,

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unexpectedly, a high level of *in vivo* expression of a
transgene can be achieved using a vector that lacks an
intron. Such vectors therefore are of particular
interest ·for *in vivo* transfection.

5   Downstream from and under control of the
transcriptional and/or translational initiation
regulatory regions is a multiple cloning site for
insertion of a nucleic acid sequence of interest which
will provide for one or more alterations of host

10 phenotype, for example to dramatically improve the
treatment of acquired immune deficiency syndrome (AIDS),
cystic fibrosis, cancer, heart disease, autoimmune
diseases and a variety of life threatening infections.
Conveniently, the multiple cloning site can be employed

15 for a variety of nucleic acid sequences in an efficient
manner. The nucleic acid sequence inserted in the
cloning site can encode a ribozyme sequence or encode a
polypeptide, for example, a protein having enzymatic
activity, with the proviso that if the coding sequence

20 encodes a polypeptide, it should lack cryptic splice
sites which can block production of appropriate mRNA
molecules and/or produce aberrantly spliced or abnormal
mRNA molecules. The polypeptide can be one which is
active intracellularly, a transmembrane protein, or it

25 may be a secreted protein. It also can be a mutant
protein, for example, which is normally secreted, but
which has been altered to act intracellularly. The
nucleic acid sequence can be DNA; it also can be a
sequence complementary to a genomic sequence (an

30 "antisense sequence"), where the genomic sequence may be
one or more of an open reading frame, an intron, a non-
coding leader sequence, or any other sequence where the
complementary sequence will inhibit transcription, or
messenger RNA processing, for example splicing, or

35 translation.

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For the treatment of AIDS, anti-TAT, REV, TAR or
other critical anti-HIV sequences may be used,
particularly for expression of the appropriate coding
sequences in T lymphocytes, macrophages and monocytes
5 which can be achieved following iv administration of the
appropriate coding sequences. Thus, DNA sequences which
code for mRNA (an antisense or ribozyme sequence such as
one to HIV-REV or a BCR-ABL sequence) or code for
proteins such as transdominant negative mutants which
10 specifically prevent the integration of HIV genes into
the host cell genomic DNA, replication of HIV sequences,
translation of HIV proteins, processing of HIV mRNA, or
virus packaging in human cells can be used.

Expression of wild-type conductance regulator
15 (CFTR) gene in the lungs of cystic fibrosis patients can
be used in the treatment of cystic fibrosis (see Collins,
(1992) *Science* 256:774-783). CFTR cDNA can be obtained
from Dr. Collins at University of Michigan or Dr. Tsui at
Toronto Sick Children's Hospital.

20 Expression of wild-type p53 in tumors of cancer
patients with absent or aberrant expression of this gene
can be used as a means of treating these patients. p53
is obtainable from Dr. Vogelstein at John Hopkins Univ.
Other methods of cancer treatment include transcription
25 of antisense sequences to over-expressed, transforming
oncogenes, such as myc or ras in tumors.

Viral pneumonias remain a major cause of death and
disability of the very young and in the aged population.
Proteins such as granulocyte macrophage colony
30 stimulating factor (GM-CSF), which stimulates production
of white blood cells from the bone marrow of
immunocompromised patients can be useful in controlling
the course of viral infections. In particular, GM-CSF
delivered via gene transfer would serve with prophylactic
35 modalities for those at risk for occurrence of viral

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pneumonia, as well as in therapeutic modalities for
treatment of the infection. GM-CSF also has significant
anti-tumor activity.

　　　　Other examples of nucleic acid sequences of
5 interest, include those encoding the LDL (low density
lipoprotein) receptor, which specifically lowers serum
cholesterol, and which can reduce the risk of heart
attack in individuals with elevated serum cholesterol
levels; sequences such as an antisense IL-1 receptor
10 sequence to down-regulate the IL-1 receptor as a
treatment of conditions involving inflammation, including
sepsis, rheumatoid arthritis and asthma; downregulation
of the gene coding for apo(a), circulating levels of
which are correlated with the risk of coronary artery
15 disease (CAD); and genes which block activity of
activated T cell clones which attack myelin in multiple
sclerosis or other targets in autoimmune diseases. A
T-cell lymphocyte clone activated to recognize and attack
myelin can be targeted by using an antisense sequence,
20 ribozyme sequence or transgene coding for a transdominant
negative mutant which specifically blocks surface
expression on the T-cell of T-cell receptor components
which mediate recognition and/or attack of myelin-
sheathed cells. Other beneficial therapeutic nucleic
25 acid sequences which can be expressed in appropriate
cells in vivo using this invention include nucleic acid
sequences encoding molecules which have superoxide
dismutase activity or catalase activity to protect the
lung from oxidant injury; endothelial prostaglandin
30 synthase to produce prostacyclin and prostaglandin E2;
and antiprotease alpha-1 antitrypsin, and erythropoietin.

　　　　The termination region which is employed
primarily will be one of convenience, since termination
regions appear to be relatively interchangeable. The
35 termination region may be native to the intended nucleic

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acid sequence of interest, or may be derived from another
source.   Convenient termination regions are available and
include the 3' end of a gene terminator and
polyadenylation signal from the same gene from which the
5 5' regulatory region is obtained.   Alternatively, a
terminator and polyadenylation signal from a different
gene or genes may be employed with similar results.
Specific sequences which regulate post-transcriptional
mRNA stability may optionally be included.   For example,
10 certain polyA sequences (Volloch and Housman, (1981) Cell
23:509-514) and ß-globin mRNA elements can increase mRNA
stability, whereas certain AU-rich sequences in mRNA can
decrease mRNA stability (Shyu et al., (1989) Genes and
Devel. 3:60-72).   In addition, AU regions in 3' non-
15 coding regions may be used to destabilize mRNA if a short
half-life mRNA is desirable for a particular application.
        The construct may additionally include sequences
for selection, such as a neomycin resistance gene or a
dihydrofolate reductase gene and/or signal sequences to
20 generate recombinant proteins that are targeted to
different cellular compartments or secreted when the wild
type sequence is not.   Any of a variety of signal
sequences may be used which are well-known to those
skilled in the art.   These signal sequences may allow
25 generation of new vaccine strategies or produce soluble
antagonists directed against specific cell surface
receptors such as transformed oncogenes.   The sequences
for selection may be on a separate plasmid and
cotransfected with the plasmid carrying the therapeutic
30 nucleic acid.   Where a carrier is used, the selection
plasmid may be complexed to a different carrier or to the
same carrier as the therapeutic plasmid.
        In some cases, it may be desirable to use
constructs that produce long term transgene effects in
35 vivo, either by integration of the transgene into host

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cell genomic DNA at high levels or by persistence of the
transgene in the nucleus of cells *in vivo* in stable,
episomal form.   When desired, integration of the
transgene into genomic DNA of host cells *in vivo* may be
5   facilitated by administering the transgene in a
linearized form (either the coding region alone, or the
coding region together with 5' and 3' regulatory
sequences, but without any plasmid sequences present).
It may be possible to further increase the incidence of
10 transgene integration into genomic DNA by incorporating a
purified retroviral enzyme, such as the HIV-1 integrase
enzyme, into the lipid carrier-DNA complex.   Appropriate
flanking sequences are placed at the 5' and 3' ends of
the transgene DNA.   These flanking sequences have been
15 shown to mediate integration of the HIV-1 DNA into host
cell genomic DNA in the presence of HIV-1 integrase.
Alternatively, duration of transgene expression *in vivo*
can be prolonged by the use of constructs that contain
non-transforming sequences of a virus such as Epstein-
20 Barr virus, and sequences such as oriP and EBNA-1, which
appear to be sufficient to allow heterologous DNA to be
replicated as a plasmid in mammalian cells (Burhans, *et
al.*, (1986) *Cell* 62:955-965).

        The recombinant coding-sequence flanked at its 5'
25 end by the promoter and regulatory sequences and at its
3' end by a terminator and regulatory sequences may be
introduced into a suitable cloning plasmid (e.g., pUC18,
pSP72) for use in direct DNA uptake in host cells
following introduction into the host.   It is a theory of
30 the invention that the naked nucleic acid when introduced
into the host is protected from degradation by nucleases
by associating with lipid carriers in the blood, such as
chylomicrons.   Thus, more effective transfection may be
achieved when lipid in the blood is at an elevated level
35 after ingestion of food.

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The nucleic acid construct also may be complexed with a carrier such as a lipid carrier, particularly a cationic lipid carriers to form a complex. By a complex is intended an association between a nucleic acid
5   construct such as a plasmid containing an expression cassette or a transcriptional cassette and a lipid mixture. The physical form of the complex may be a liposome with nucleic acid complexed to the outside or entrapped within the liposome, or the complex may be in
10 the form of interleavened lipid and nucleic acid, or the complex may be a mixture of any or all of the above physical forms. For intravenous administration, generally the complex is prepared by sonicating the lipid mixture to be used and then mixing the sonicated mixture
15 with the nucleic acid in an appropriate DNA:lipid ratio in a physiologically acceptable diluent immediately prior to use.   The lipid carriers can be prepared from a variety of cationic lipids, including DOTAP, DOTMA, DDAB, L-PE, and the like. Lipid carrier mixtures containing a
20 cationic lipid, such as N-[1-(2,3-dioleyloxy) propyl]-N,N,N-triethylammonium chloride (DOTMA) also known as "lipofectin", dimethyl dioctadecyl ammonium bromide (DDAB), 1,2-dioleoyloxy-3-(trimethylammonio) propane (DOTAP) or L-lysinyl-phosphatidylethanolamine (L-PE) and
25 a second lipid, such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol (Chol), are of particular interest. DOTMA synthesis is described in Felgner, *et al.*, (1987) *Proc. Nat. Acad. Sciences, (USA)* 84:7413-7417. DOTAP synthesis is described in Stamatatos, *et al.*,
30 *Biochemistry*, (1988) 27:3917-3925. DOTMA:DOPE lipid carriers can be purchased from, for example, BRL. DOTAP:DOPE lipid carriers can be purchased from Boehringer Mannheim. Cholesterol and DDAB are commercially available from Sigma Corporation. DOPE is
35 commercially available from Avanti Polar Lipids.

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DDAB:DOPE can be purchased from Promega. Biodegradable
cationic amphophiles also can be used (see, for example,
copending PCT application attorney docket number MEBI-
002/00WO filed December 17, 1992).

5   Lipid carriers such as cationic liposomes can
mediate high level cellular expression of transgenes or
mRNA by delivering the nucleic acid into a wide variety
of cells in culture. The use of specific cationic lipids
can confer specific advantages for in vivo delivery of
10 complexes. For example, iv injection of nucleic acid
complexed to DOTAP-containing liposomes or ethyl-
phosphatidylcholine (E-PC) lipid carriers can target
transgene expression primarily to the lung. Furthermore,
DOTAP, as well as L-PE and cholesterol ester β-alanine
15 (CEBA) are fully metabolized by cells, whereas DOTMA
cannot be fully metabolized by cells. Therefore, DOTAP,
E-PC, and L-PE, but not DOTMA, are suitable for repeated
injection into mammalian hosts. Additionally, using a
lipid carrier comprising a cationic lipid and a second
20 lipid, particularly cholesterol or DOPE can maximize
transgene expression in vivo. Also, mixing a steroid,
such as cholesterol, instead of DOPE, with DOTAP, DOTMA,
or DDAB, substantially increases transgene expression in
vivo.

25   Particular cells and tissues can be targeted,
depending upon the route of administration and the site
of administration. For example, a tissue which is
closest to the site of injection in the direction of
blood flow can be transfected in the absence of any
30 specific targeting. Additionally, if desired, the lipid
carriers may be modified to direct the complexes to
particular types of cells using site-directing molecules.
Thus antibodies or ligands for particular receptors or
other cell surface proteins may be employed, with a
35 target cell associated with a particular surface protein.

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For example, the AIDS virus is primarily directed to
cells having the CD4 surface protein. By having anti-CD4
antibody bound to the surface of the lipid carrier, a
nucleic acid lipid carrier complex can be directed
5 primarily to T-helper cells.

     A particular ligand or antibody can be conjugated
to the lipid in accordance with conventional ways, either
by conjugating the site-directing molecule to a lipid or
by providing for a linking group on a lipid present in
10 the bilayer for linking to a functionality of the site-
directing compound. Such techniques are well known to
those skilled in the art. Ligand-directed DNA-polycation
complexes have been shown to transfect to hepatocytes in
the liver after iv injection; the ability to transfect
15 other cell types or tissue types by this approach has not
been demonstrated.

     Non-cationic lipid carriers, particularly pH
sensitive liposomes, offer another potentially attractive
approach to *in vivo* gene therapy. However, as compared
20 to cationic liposomes, pH sensitive liposomes are less
efficient in capturing DNA and delivering DNA
intracellularly and may be inactivated in the presence of
serum, thus limiting their iv use.

     A number of factors can affect the amount of
25 expression in transfected tissue and thus can be used to
modify the level of expression to fit a particular
purpose. Where a high level of expression is desired,
all factors can be optimized, where less expression is
desired, one or more parameters can be altered so that
30 the desired level of expression is attained. For
example, if high expression would exceed the therapeutic
window, then less than optimum conditions can be used.
The factors which can be modified are as follows:

     Either the lipid composition of the complex or the
35 mean diameter of the lipid carriers (when in particle

form such as a liposome) injected can dramatically affect the level of transgene expression produced *in vivo*.

Thus, the liposomal lipid compositions generally have a composition of 50% molar ratio of cationic lipid to non-
5 cationic lipid, but may range from 5% to 100%. The diameter of the lipid carriers should generally be within the range of 100nm to 10 μm. Cationic lipid carrier-DNA complexes wherein the lipid carriers range from 100 nm to several μm in diameter can produce significant levels of
10 transgene expression after systemic introduction into a mammalian host.

The use of lipid carriers of greater than 500 nm (i.e. multilamellar vesicles (MLV) or large unilamellar vesicles (LUV)) can in certain cases significantly
15 increase the level of transgene expression achieved in a mammalian host when compared to small unilamellar vesicles (SUV). MLV and LUV are prepared by vortexing rather than sonicating after addition of the aqueous material to the dry lipid film. Where it is desired to
20 use particles, the resulting lipid carriers can be extruded under high pressure through sized polycarbonate membranes to achieve particular uniform size distributions for a particular use.

The use of particular nucleic acid to lipid
25 carrier ratio also can increase the amount of transfection and/or the level of transcription and/or expression of a nucleic acid sequence of interest. The ratios used determine whether and to what level transgenes are expressed *in vivo* thus can be optimized,
30 depending upon various factors including the nature of the construct, the size and lipid composition of the lipid carrier and whether it is MLV or SUV, the route of administration and the host mammal. As an example, using a reporter gene CAT (chloramphenicol acetyl transferase),
35 an approximately 1:1 (range 0.5:1 to 2:1) DNA to lipid

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carrier ratio (μg DNA to nmoles of the cationic lipid) produces the highest levels of gene expression in a mouse in all organs after ip administration, and an approximately 1:4 ratio, (range 2:1 to 1:7) produces the
5 highest levels of gene expression in all organs after iv administration. In addition to achieving a high level of transgene expression in a wide variety of tissues using optimal conditions, the majority of all cells present in the lung, spleen, lymph nodes and bone marrow are
10 transfected *in vivo*, as well as the majority of all endothelial cells present in the heart.

The DNA:lipid carrier ratio also can affect at what level transgenes are expressed in mammalian hosts after systemic injection of the complexes. Several
15 factors are important in order optimize the DNA:lipid carrier ratio for a particular expression level desired. Thus, specific DNA:lipid carrier ratios are required for each type of cationic lipid used as well as for each different lipid carrier size used. To optimize for
20 maximum expression, for each lipid carrier composition used, DNA is be mixed together with the lipid carriers in multiple different ratios, ranging from 6:1 to 1:10 (μg DNA to nmole cationic lipid), in order first to determine which ratios result in aggregation of the DNA:lipid
25 carrier complexes. Ratios which result in aggregation cannot be used *in vivo*. The ratios which do not result in aggregation are tested in animal models to determine which of the DNA:lipid carrier ratios confers the desired level of transgene expression *in vivo*. For example, the
30 optimal DNA:lipid carrier ratios for SUV for DOTMA:DOPE, DDAB:DOPE, DOTAP:DOPE, DOTAP:Cholesterol, L-PE:CEBA, DDAB:Cholesterol and L-PE:DOPE are 1:4, 1:3, (very low activity at all ratios), 1:6, 1:1, 1:5, and 2:1, respectively. DNA:lipid carrier complexes are made in
35 appropriate physiologic solutions. The DNA:lipid carrier

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complexes are mixed in physiologic solutions
(approximately 290 milliosmoles) which do not themselves
induce aggregation of the DNA:lipid carrier complexes.
The solutions include 5% dextrose in water or normal

5   saline. Cell surface receptors for cationic lipid
carriers can be used to both regulate and confer target
cell specificity on transgene expression in mammalian
hosts. Cationic lipid carrier:DNA complexes are
internalized by cells by a classical receptor-mediated

10   endocytosis (see Figure 7) using cell surface receptors
which contain specific binding sites for, and are able to
internalize, cationic molecules. Using agents such as
cytokines, growth factors, other soluble proteins and
certain drugs, it is thus possible to selectively up or

15   down regulate these cation-binding receptors. The rate
of up or down regulation of these receptors by the
appropriate agent will allow selection of specific cells
for enhanced or reduced levels of transfection in vivo.
Cell surface receptors for naked DNA also can be used

20   both to regulate and to confer target cell specificity on
transgenic expression in mammalian host.
          The most frequent interaction between DOTMA lipid
carriers, either uni- or multilamellar lipid carriers,
complexed to plasmid DNA and the various cell types (for

25   example, CV-1 monkey kidney cells, U937 human
myelomonocytic leukemia cells, K562, MEL (murine
erythroblastic leukemia cells), rat alveolar macrophages,
and alveolar type II cells), is that of lipid carrier
adhesion and internalization. This interaction is common

30   to well-defined examples of receptor-mediated
endocytosis. All cells which appear to have contacted
cationic lipid carrier:DNA complexes ingest the complexes
following binding of the complexes to the plasma
membrane. All these cell types demonstrate the same

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classical receptor-mediated endocytic pathway of
internalization.

The mammalian host may be any mammal, particularly
a mammal having symptoms of a genetically-based disorder
5   or an infectious disease which is amenable to gene-based
therapy. Thus, the subject application finds use in
domestic animals, feed stock, such as bovine, ovine, and
porcine, as well as primates, particularly humans. The
mammalian host may be pregnant, and the intended
10   recipient of the gene-based therapy may be either the
gravid female or the fetus or both.

In the method of the invention, transfection *in
vivo* is obtained by introducing a therapeutic
transcription or expression vector into the mammalian
15   host, either as naked DNA or complexed to lipid carriers,
particularly cationic lipid carriers. The constructs may
provide for integration into the host cell genome for
stable maintenance of the transgene or for episomal
expression of the transgene. The introduction into the
20   mammalian host may be by any of several routes, including
intravenous or intraperitoneal injection,
intratracheally, intrathecally, parenterally,
intraarticularly, intranasally, intramuscularly, topical,
transdermal, application to any mucous membrane surface,
25   corneal instillation, etc. Of particular interest is the
introduction of a therapeutic expression vector into a
circulating bodily fluid or into a body orifice or
cavity, such as lung, colon, vagina, and the like. Thus,
iv administration and intrathecal administration are of
30   particular interest since the vector may be widely
disseminated following such routes of administration, and
aerosol administration finds use with introduction into a
body orifice or cavity. Any physiologically acceptable
medium may be employed for administering the DNA or lipid
35   carriers, such as deionized water, saline, phosphate-

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buffered saline, 5% dextrose in water, and the like,
depending upon the route of administration   Other
components may be included in the formulation such as
buffers, stabilizers, biocides, etc. These components
5 have found extensive exemplification in the literature
and need not be described in particular here. Any
diluent or components of diluents that would cause
aggregation of the complexes should be avoided, including
high salt, chelating agents, and the like.
10   The amount of naked DNA or complexes used will be
an amount sufficient to provide for adequate
dissemination to a variety of tissues after entry of the
DNA or complexes into the bloodstream and to provide for
a therapeutic level of expression in transfected tissues.
15 A therapeutic level of expression is a sufficient amount
of expression to, prevent, treat or palliate a disease or
infection of the host mammal. In addition, the dose of
the nucleic acid vector used must be sufficient to
produce a desired level of transgene expression in the
20 tissue or tisses of interest, *in vivo* for example, $\geq$ 1mg
of an expression plasmid alone injected into a mouse
results in a high level of expression of the CAT gene in
multiple tissues. Other DNA sequences, such as
adenovirus VA genes can be included in the administration
25 medium and be co-transfected with the gene of interest.
The presence of genes coding for the adenovirus VA gene
product may significantly enhance the translation of mRNA
transcribed from the plasmid if this is desired.
          The level and tissues of expression of the
30 recombinant gene may be determined at the mRNA level
and/or at the level of polypeptide or protein. Gene
product may be quantitated by measuring its biological
activity in tissues. For example, enzymatic activity can
be measured by biological assay or by identifying the
35 gene product in transfected cells by immunostaining

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techniques such as probing with an antibody which
specifically recognizes the gene product or a reporter
gene product present in the expression cassette.
Alternatively, potential therapeutic effects of the gene
5   product can measured, for example where the DNA sequence
of interest encodes GM-CSF, by determining the effects of
gene expression on survival of lethally irradiated
animals in which the GM-CSF transgene is expressed.
Production of significant amounts of a transgene product
10 will substantially prolong the survival of these mice.
        Where expression of the polypeptide/protein or
even the mRNA itself confers a changed biochemical
phenotype upon the host, the presence of a new phenotype
or absence of an old phenotype may be evaluated; for
15 example, as a result of transfection of the host cells,
there may be enhanced production of pre-existing
desirable products formerly produced in insufficient
quantities or there may be reduction or even suppression
of an undesirable gene product using antisense, ribozyme
20 or co-suppression technologies; in the case of
suppression, a reduction of the gene product may be
determined. Typically, the therapeutic cassette is not
integrated into the host cell genome. If necessary, the
treatment can be repeated on an ad hoc basis depending
25 upon the results achieved. If the treatment is repeated,
the mammalian host can be monitored to ensure that there
is no adverse immune or other response to the treatment.

        As an example, in a clinical setting where it is
30 desired to treat a particular disease state, both the
biological efficacy of the treatment modality as well as
the clinical efficacy need to be evaluated, if possible.
For example, in the treatment of cystic fibrosis, there
is s generalized epithelial dysfunction which manifests
35 itself as abnormalities in the electrolyte and water

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content of luminal liquid or "secretions" of the airways,
sweat glands, intestinal and reproductive tracts, and
pancreas. The biological efficacy of gene therapy
therefore can be evaluated by for example measuring the
5   transepithelial electrical potential difference prior to
treatment and following transaction. The evaluations can
be done following transfection of nasal cells and also
following transfection lung cells. Examples of
techniques which can be used for measuring the
10 bioelectric potential difference across respiratory
epithelial and cystic fibrosis as described in Knowles,
et al., (1981) *New England General of Medicine* 305:1489-
1495; Knowles, et al., (1983) *General of Clinical
Investigation* 71:1410-1417; and Knowles, et al., (1983)
15 *Science* 221:1067-1070. The clinical efficacy, whether
treatment of the underlying defect is effective in
changing the course of disease, can be more difficult to
measure. While the evaluation of the biological efficacy
goes a long way as a surrogate end point for the clinical
20 efficacy, and is not definitive. Thus, measuring a
clinical endpoint such as the so-called "spirometry"
factors which give an indication of lung function of a
for example, a six-month period of time, may give an
indication of a clinical efficacy of the treatment
25 regimen. Typical measurements would include forced vital
capacity (FVC) of the lung and forced expired volume in
one second (FEV). An example of the type of clinical
study which can be performed to evaluate the efficacy of
gene therapy for cystic fibrosis is one which is being
30 used for amiloride for the treatment of lung disease and
cystic fibrosis: Knowles, et al., (1990) *New England
General of Medicine* 322:1189-1194. Similarly, one
skilled in the art can evaluate the biological and
clinical efficacy of a particular gene therapy protocol.

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The subject compositions can be provided for use in one or more procedures. Kits will usually include the DNA either as naked DNA or complexed to lipid carriers. Additionally, lipid carriers may be provided in a

5   separate container for complexing with the provided DNA. The DNA either for direct administration or for complexing with lipid carriers, or the lipid carrier/DNA complexes may be present as concentrates which may be further diluted prior to use or they may be provided at

10 the concentration of use, where the vials may include one or more dosages. Conveniently, single dosages may be provided in sterile vials so that the physician or veterinarian may employ the vials directly, where the vials will have the desired amount and concentration of

15 agents. Thus, a vial may contain the DNA or the DNA/lipid carrier complexes in appropriate proportional amounts. When the vials contain the formulation for direct use, usually there will be no need for other reagents for use with the method.

20   The invention finds use in *in vivo* prevention, treatment and/or palliation of a number of diseases. *In vivo* replacement of a gene can be accomplished by techniques such as homologous recombination or initial knockout of the aberrant gene and subsequent replacement

25 with the desired transgene. An additional benefit of expressing nucleic acids in appropriate cells *in vivo* using this invention, is that the encoded proteins would be processed and subject to post translational modification in the correct manner.

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The following examples are offered by way of
illustration and not by limitation.

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Example 1
Preparation of Plasmids for *in vivo* Gene Therapy

Details regarding the plasmids that have been used
for transfection of mammalian cells are as follows.

5   pRSVCAT: construction of this plasmid is described
in Gorman *et al.*, (1982) *Proc. Nat. Acad. Sciences (USA)*
79:6777-6781. In the pRSVCAT plasmid, the 3'-RSVLTR is
juxtaposed as a promoter upstream from CAT encoding
sequences. The distance between the LTR transcriptional

10 start site and the CAT initiation codon (the first AUG
downstream from the start site) is about 70 bp.

p5'PRL3-CAT: construction of this plasmid is
described in Sakai

et al., (1988) *Genes and Development* 2:1144-1154.

15   pSIS-CAT: construction of this plasmid is
described in Huang and Gorman, *Nucleic Acids Research*,
(1990) 18:937-948.

pZN20: construction of this plasmid is illustrated
in Figure 5. The plasmid was prepared as follows.

20 pCATwt760 (Stinski and Roehr, (1985) *J. Virol.* 55:431-
441) was treated with *Hind*III and the fragment containing
the HCMV IE 1 enhancer and promoter element purified.
The isolated fragment was then cloned into the *Hind*III
site of pSP72 (Promega) creating pZN9. Clones were

25 screened in which the enhancer and promoter element is as
shown in Figure 5. Following partial *Hind*III digestion
of pZN9, the blunt ends were filled in with DNA
polymerase I Klenow fragment. The resulting clone pZN12
had lost the *Hind*III site 5' to the enhancer and promoter

30 element. pZN12 was then treated with *Nco*I and *Hind*III
and the large *Nco*I-*Hind*III fragment purified and ligated
to a purified small *Nco*I-*Hind*III fragment from
pBC12/CMV/IL-2 (Cullen, (1986) *Cell* 46:973-982.
pBC12/CMV/IL-2 contains the HCMV promoter from the AD169

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strain. The resulting clone was pZN13. pZN13 was partially digested with *Bam*HI, filled in with DNA polymerase I Klenow fragment and the resulting clones screened for the clone which had lost the *Bam*HI site at

5   the 5' end of the enhancer and promoter element. The resulting clone was called pZN17. pZN17 was treated with *Hind*III and *Bam*HI and the resulting *Hind*III-*Bam*HI large fragment was purified and ligated to a purified small *Hind*III-*Bam*HI fragment obtained from pSV2-CAT (Gorman, *et*

10   *al.,* (1982), *Molecular Cell Biology* 2:1044-1051). The resulting clone was pZN20. The full restriction map of HCMV (Towne) is shown in Figure 6A. HCMV (AD169) is shown in Figure 6C. A comparison of the two promoters is shown in Figure 6B. Significantly more expression is

15   obtained when a promoter from the AD169 strain is used as compared to one from the Towne strain. pZN20 contains a composite promoter which has the Towne sequence 5' of the *Nco*I site and the AD169 sequence 3' of the *Nco*I site. The *Nco*I site is indicated by the asterisk in Figure 6B.

20   pZN20 has this composite HCMV promoter followed by the CAT gene, SV40 t-intron and SV40 polyA adenylation site.

pZN27: Construction of this plasmid is illustrated in Figure 9. pZN27 contains the composite HCMV promoter followed in order by the SV40 t-intron, the

25   CAT coding sequence and the SV40 polyA adenylation site.
pZN46: Construction of this plasmid is shown in Figure 10A and Figure 10B. pZN46 contains the composite HCMV promoter, followed by the human IL-2 gene, rat preproinsulin 2 intron and polyA addition site from the

30   rat preproinsulin 2 gene. These last three components were derived from the pBC12/CMV/IL-2 plasmid of Cullen (*Cell,* (1986) 46:973-982. The rat preproinsulin 2 intron was modified by deleting an internal 162 base pair *Nde*I fragment.

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pZN32: Construction of this plasmid is shown in
Figure 11. pZN32 contains the composite HCMV promoter
followed in order by the modified rat preproinsulin 2
intron described for pZN46, human CFTR cDNA, and rat
5 preproinsulin 2 gene polyA addition site as described for
pZN46. CFTR cDNA was obtained from pBQ4.7 from F.
Collins (Univ. of Michigan).

pZN51: Construction of this plasmid is shown in
Figure 13. pZN51 contains the composite HCMV promoter
10 followed by the CAT coding sequence and the SV40 polyA
adenylation site.

pZN60, pZN61, pZN62, pZN63: Construction of these
plasmids is shown in Figure 14. pZN60 contains the HCMV
composite promoter followed by the modified rat
15 preproinsulin 2 intron, the CAT coding sequence, and the
SV40 polyA addition site. pZN61 is identical to pZN60
but contains an additional 166 base pairs 5' to the
intron. This additional DNA is the 166 base pairs
immediately 5' of the intron in the pBC12/CMV/IL-2
20 plasmid and contains rat preproinsulin 2 gene coding
sequence. pZN62 is similar to pZN60 except that the
intron is 3' of the CAT coding sequence rather than 5' as
in pZN60. pZN63 is identical to pZN62 except for the
additional 166 base pairs 5' to the intron. This is the
25 same additional sequence described for pZN61.

pCIS-CAT: This plasmid was made as described in
Huang, M.T.F. and Gorman, C.M., (1990) *Nucl. Acids Res.*
18:937-947, with the exception that a CMV promoter and a
hybrid intron sequence were used rather than the SV40
30 promoter in the plasmid pML.I.CAT, Gorman, *et al.*, (1990)
DNA Protein Eng. Tech., 2:3-10.

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Example 2
Preparation of Lipid carriers and DNA Complexing with Lipid carriers

Lipid carriers containing a cationic lipid, such
5   as N-[(1-2-3-dioleyloxy) propyl]
-N,N,N-triethylammonium chloride (DOTMA), dimethyl
dioctadecyl ammonium bromide (DDAB), or
1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP) or L-
lysinyl-phosphatidylethanolamine (L-PE) and a second
10 lipid, such as dioleoylphosphatidylethanolamine (DOPE) or
cholesterol, were prepared as follows.

Preparation of lipid carriers:
Lipids, e.g. DDAB, L-PE, cholesterol-ester-β-
alanine (CEBA), DOTAP, and cholesterol (Chol) were
15 dissolved in chloroform. Proper amounts of each lipid
(determined by the desired molar ratio of each lipid in
the final lipid carrier formulation usually 1 to 1 moles
cationic lipid to moles non-cationic lipid but ranging
from 5 to 1 up to 1 to 5) were mixed together and
20 evaporated to dryness on a rotary evaporator. The lipid
film was then resuspended by vortexing after the addition
of 5% dextrose in water or lipid carrier buffer (25 mM
Tris-HCl pH 7.4, 100 μM ZnCl$_2$, NaCl to isotonicity. To
make a final lipid concentration of 20mM of multi-
25 lamellar vesicles (MLV). For the preparation of small
unilamellar vesicles (SUV), the mixture was then
sonicated in a bath sonicator for 15 min, and the lipid
carriers were stored under argon at 4°C until use.

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<u>Plasmid Preparation:</u>

The *E. coli* strain HB101 which carried the plasmid
was grown in TB at 37°C. The method of plasmid
purification is a modification of the protocol of "lysis
5   by alkali" and "purification of plasmid DNA by
precipitation with polyethylene glycol" described by
Sambrook *et al.*, (1989) (*Molecular Cloning*, 2nd edition,
Cold Spring Harbor Laboratory Press). The modification
was that the precipitation of DNA by PEG was omitted.
10   The final DNA preparation was dissolved in 10mM Tris-HCl
pH 8.0.

<u>Preparation of lipid carrier-plasmid complexes:</u>

Plasmids were diluted separately in 5% dextrose in
water solution to the desired concentration (usually 1
15   μg/μl). The lipid carriers were also diluted in 5%
dextrose in water to the same volume as the plasmid.

The amounts of lipid carriers used were determined
based on the ratio of nmoles of lipid to μg of plasmid
added, e.g. for lipid carrier:plasmid=1:1, one nmole of
20   cationic lipid is mixed with 1 μg of plasmid DNA.
Plasmid and lipid carriers were then mixed together to
form DNA:lipid carrier complexes.
<u>Dose injected</u>. At least 50 μg, and routinely 100 μg of
plasmid DNA complexed to cationic lipid carriers were
25   injected intravenously by tail vein per mouse. For
injection of plasmid alone, at least 500 μg and routinely
2 mg of plasmid DNA were injected intravenously by tail
vein per mouse.

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Example 3
Demonstration by Immunohistochemistry of CAT Gene
Expression in the
Lung After Intravenous (iv) injection of pZN27-DDAB:
5   Cholesterol Lipid carrier Complexes

Lipid carrier: DDAB:Cholesterol=1:1, stock 20 mM in
lipid carrier buffer.
Plasmid: pZN27.
DNA:Lipid carrier Ratio: lipid carrier:plasmid=5
10 nanomoles cationic lipid:1 μg DNA
DNA dose: 100 μg plasmid DNA in 200 μl 5% dextrose in
water was injected iv by tail vein per mouse.
Mice: ICR, female, 25 grams.

Immunohistochemical staining to detect CAT protein in
15 lung sections of mice treated in vivo.
Procedure: Forty eight hours after injection of the
pZN27-DDAB:Cholesterol complexes, the lungs are removed,
perfused with 33% O.C.T. Embedding Medium (Miles, Inc.),
embedded in O.C.T. and snap frozen. Frozen tissues were
20 sectioned at 6 μm, collected onto glass slides, fixed for
10 minutes in 4°C acetone and then placed in 0.2% Triton
X-100 to permeabilize membranes. Sections were then
incubated for 12-48 hours with the monoclonal anti-CAT
antibody (available from Dr. Parker Antin, Univ. of
25 Arizona) or isotype negative control antibody at the
appropriate dilution. After washing, a biotinylated
antibody directed against the primary antibody (Zymed, S.
San Francisco) was added for a minimum of 60 minutes,
followed by application of the streptavidin-alkaline
30 phosphatase complex (Zymed) for 60 minutes. The
substrate-chromogen appropriate for the enzyme label was
then applied, per the manufacturer's instructions.

Slides were coverslipped in water-soluble mounting media
for examination.

Results: The results are shown in Figure 1 and
demonstrate diffuse staining of the lung. The stain
5   localizes to the alveolar walls, indicating that greater
than 70% of pulmonary vascular endothelial cells, as well
as alveolar lining cells, including type I and type II
cells and alveolar macrophages are transfected by a
single iv injection of DNA lipid carrier complexes. In
10 addition, significant numbers of bronchiolar airway
lining cells stain positively for CAT protein, and are
therefore transfected in vivo by iv injection of lipid
carrier:DNA complexes. Thus, the great majority of all
cells in the lung transfected by an iv injection of
15 pZN27-DDAB:Cholesterol complexes.

                        Example 4
                   Expression of pCIS-CAT
          Following Intraperitoneal Administration

     Effect of the Amount of pCIS-CAT-cationic lipid carrier
20   complexes injected ip on the level of CAT gene expression
     in vivo.
          Female ICR mice were injected ip with 1 ml of 5%
dextrose in water containing 0.01, 0.1, or 1 mg of pCIS-
CAT expression plasmid complexed with 0.01, 0.1 or 1
25 μmoles, respectively, of DDAB:DOPE lipid carriers. Mice
were sacrificed 48 hours later, the organs removed, and
tissues were homogenized in 0.25M Tris-HCl buffer pH 7.8,
using a hand-held homogenizer. Cytoplasmic extracts were
made, normalized by protein content and the level of CAT
30 activity then measured. The experiments comprise three
animals per group and the results show the mean dpm ± SEM
of acetylated chloramphenicol.

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Methods: Lipid carriers containing DDAB were prepared in 1:1 molar ratio with DOPE, as follows: 10 μmoles of DOPE dissolved in chloroform and 10 μmoles of the cationic lipid, dissolved in ethanol were evaporated
5 to dryness on a rotary evaporator. One ml sterile of water was added, and the mixture was sonicated in a bath sonicator (Laboratory Supply, Hicksville, NY) for 20 min. Lipid carriers had mean diameters of approximately 100 $\pm$25 nm. For CAT assay, cell extracts were made, and
10 their protein content determined by the Coomassie Blue assay (BioRad, Richmond). One hundred μg of protein from the lung, spleen, liver, and heart extracts, and 50 μg of lymph node extract were reacted with ^{14}C labeled chloramphenicol and chromatographed as previously
15 described (Gorman, *supra*). To calculate dpm, both the acetylated and unacetylated species were cut from TLC plates and radioactivity counted in a scintillation counter.

　　　　Results: To assess potential dose-response
20 relationships *in vivo*, animals were injected animals in groups of three with 0.01 mg, 0.1 mg, or 1 mg of pCIS-CAT plasmid complexed with 0.01 μmole, 0.1 μmole, or 1 μmole respectively of DDAB:DOPE lipid carriers. Both the 0.1 mg and 1 mg DNA doses produced highly significant levels
25 of CAT protein ($p < 0.005$) in all the organs assayed. Maximal levels of CAT gene expression in each organ were produced by the 1 mg DNA dose: increasing the DNA-lipid carrier dose 10 fold resulted in an approximately 2 fold increase in lymph node CAT levels and a 3 fold increase
30 in the spleen.

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Example 5
Demonstration of CAT gene expression in the spleen after intravenous (iv) injection of p5'PRL3-CAT:L-PE:CEBA complexes.

5   Lipid carrier: L-PE:CEBA=1:1, stock 20 mM in lipid
    carrier buffer.
    Plasmid: p5'PRL3-CAT.   (Sakai, et al., (1988) Genes and
    Development 2:1144-1154)
    DNA:Lipid carrier Ratio: lipid carrier:plasmid=1 nmole
10 cationic lipid:
      1 μg plasmid DNA.
    DNA dose: 200 μg plasmid DNA in 200 μl 5% dextrose in
    water was injected by tail vein per mouse.
    Mice: Balb/c, female, 25 grams.
15   Tissue extraction procedure: Forty eight hours
    after tail vein injection, mice were sacrificed, whole
    spleens were homogenized in 1ml of 0.25M Tris-HCl pH 7.8,
    5mM EDTA, 80 μg/ml PMSF and the resulting extract was
    centrifuged and then the supernatant was subjected to 3
20 cycles of freeze-thaw and then heated to 65°C for 20 min.
            CAT assay procedure: 100 μl of extract+10 μl of
    20 mM acetyl CoA+4 μl of ^{14}C-chloramphenicol (25 μCi/ml,
    55 mCi/mmole, Amersham) were incubated together at 37°C
    for 6 hr.   At 3 hours, an additional 10 μl of acetyl CoA
25 was added.
            Results:
            This experiment showed that a significant level of
    CAT activity was present in the spleen extract of the
    treated animal, but not in the extract of control spleen,
30 taken from an animal injected with lipid carrier alone.

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Example 6
Injection of DOTMA:DOPE+pSIS-CAT Plasmid Clearly Did Not
Produce
Detectable CAT Gene Expression *in vivo*)

5 Lipid carrier: DOTMA:DOPE=1:1, in 5% dextrose in water
   Plasmid: pSIS-CAT (Huang, and Gorman, (1990) *Nucleic*
                 Acids Research 18:937-947).
   Ratio: Cationic lipid:plasmid=4 nmoles: 1 µg, dose:
                 100 µg DNA in 200 µl 5% dextrose in water.
10 Mouse: ICR, female, 25 grams.
   Injection: tail vein.
   Tissue collection and processing: Mice were sacrificed at
   day 2 and day 6, and lung, spleen, liver, and heart were
   collected. The whole organs were homogenized in 0.5 ml,
15 except livers which were homogenized in 2.0 ml, of 0.25M
   Tris-HCl pH 7.8, 5 mM EDTA, 2 µg/ml aprotinin, 1 µg/ml E-
   64, and 0.5 µg/ml leupeptin (all protease inhibitors
   purchased from Boehringer Mannheim). Extracts were
   subjected to three cycles of freeze-thaw, then heated to
20 65°C for 10 min.
   CAT assay: 100 µl of extracts for each assay with 0.3
   µCi of ^{14}C-chloramphenicol and 10 µl of 20 mM acetyl CoA
   at 37°C for either 5 hrs or 24.5 hrs, and the materials
   were then extracted using ethyl acetate and analyzed on
25 TLC plates.
   Results:
          There were no acetylated chloramphenicol species
   presented as determined by comparing the extracts from
   treated animals with that from control animals. Thus,
30 under similar experimental conditions that produce high
   level expression of pZN27, the use of the pSIS-CAT
   expression vector does not result in any detectable
   expression of the linked-CAT gene in any of the tissues
   assayed *in vivo*. The lack of expression of pSIS-CAT *in*

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vivo may be due either to a different promoter-enhancer
element (SV40) or to a different intron sequence when
compared to the pZN27 vector, which yields high level *in
vivo* expression.

5   Example 7
          Interaction Of DNA: Lipid carrier Complexes
                    With Cell Surface Receptors

          Cells and cell culture: CV-1 (African green
monkey kidney), U937 (human myelocytic leukemia), murine
10 erythroleukemia (MEL) cells, and K562 cells (human
erythroleukemia cells were obtained from the American
Type Culture Collection (Rockville, MD). CV-1 and MEL
cells were maintained in Dulbecco minimum essential
medium DME-H-21 with 5% fetal bovine serum (FBS) at 37°C
15 and 7% CO_2. Rat alveolar type II cells and rat alveolar
macrophages were isolated and purified as previously
described. (Debs *et al.*, (1987) *Amer. Rev. Respiratory
Disease* 135:731-737; Dobbs, (1986) *Amer. Rev. Respiratory
Disease* 134:141-145) Type II cells were maintained in
20 DME-H-16 with 5% FBS at 37°C and 7% CO_2. Twenty nmole of
DOTMA:DOPE lipid carriers complexed to 20 μg of pRSV-CAT
plasmid DNA were added to 2 million cells growing in 60
mm Falcon plastic dishes (either SUV or MLV), and fixed
for EM at time points from 15 minutes to 2 hours
25 thereafter.
Fixation and Processing for Electron Microscopy
          DOTMA lipid carriers and cells in tissue culture
or freshly isolated from blood or pulmonary alveoli were
fixed in 1.5% glutaraldehyde in 0.1 molar sodium
30 cacodylate buffer containing 1% sucrose, pH 7.4, at room
temperature for 1 hr. Following tannic acid and uranyl
acetate enhancement, tissue was dehydrated in a graded
series of alcohols and embedded in epoxy 812 resin

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(Ernest F. Fullam, Inc., Latham, NY) sectioned on an MT 2 microtome using diamond knives and examined with a Jeol 100CX transmission electron microscope operating at 80 kV. Electron photomicrographs of the internalization of
5 lipid complexes in CV-1 monkey kidney cells are shown in Figure 7. The arrows in panel (a) show particles binding to clathrin-coated pits; in panel (b), the arrows show where the particles have been ingested and are present in endosomes.

10   The most frequent interaction between DOTMA lipid carriers, either uni- or multilamellar lipid carriers, complexed to plasmid DNA and the various cell types (CV-1 monkey kidney cells, U937 human myelomonocytic leukemia cells, K562, MEL erythroblastic leukemia cells, rat
15 alveolar macrophages, and alveolar type II cells), is that of lipid carrier adhesion and internalization in a typical coated vesicle pathway (Fig. 7 a-f). This interaction is common to well defined examples of receptor-mediated endocytosis. All cells which appear to
20 have contacted cationic lipid carrier:DNA complexes ingest the complexes after binding to the plasma membrane. All these cell types (derived from rodent, monkey and human cells, demonstrated the same classical receptor-mediated endocytic pathway of internalization.
25 DNA-cationic liposome complexes were generally taken up by human cells as well as or better than nonhuman and particularly rodent cells of similar lineage.

Example 8

Demonstration that Mouse T Lymphocytes are Transfected in
30 vivo.

        Female ICR mice were injected ip with 1 ml of 5% dextrose in water containing 1 mg of a pZN27 plasmid complexed to 1 μmole of DDAB:DOPE (1:1 molar, SUV) lipid

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carriers. Mice were sacrificed 48 hrs. later, the spleen
and lymph nodes were removed, and rendered into single
cell suspensions by homogenizing in serum containing
medium. The cells were then incubated with FITC-
5   conjugated anti-Thy 1.2 antibody (provided by Dr. J.
Beck, San Francisco Veterans Administration Medical
Center) and sorted by FACS. The Thy 1.2$^+$ T lymphocyte
fraction was cytospun onto microscope slides and fixed
and probed for the presence of intracellular CAT protein
10 after permeabilizing the cells using 0.25% Triton X-100.
The cells were incubated with an anti-CAT monoclonal
antibody (gift from Dr. P. Antin, Univ. of Arizona) for 1
hr. at 20°C and then stained with a Texas Red- conjugated
goat-anti-mouse IgG for 1 hr. at 20°C. Figure 2A shows a
15 field of T lymphocytes by phase contrast microscopy and
2B shows the same field viewed by fluorescence
microscopy. These results demonstrate that more than 70%
of Thy 1.2$^+$ T lymphocytes are transfected in vivo (as
shown by the red fluorescence), by a single ip injection
20 of pZN27:DDAB:DOPE complexes. Thy 1.2$^+$ lymphocytes from
untreated mice do not show immunofluorescent staining as
shown in 2D; a phase contrast micrograph of the same
field is shown in 2C.

Example 9
25   Demonstration that Mouse Hematopoietic Bone Marrow-
Derived
              Cells Are Transfected in vivo.

Female ICR mice were injected ip with 1 ml of 5%
dextrose in water containing 1 mg of a pZN27 plasmid
30 complexed to 1 μmole of DDAB:DOPE SUV lipid carriers.
Mice were sacrificed 48 hrs. later, bone marrow-derived
hematopoietic cells were then obtained by perfusing the
femur cavity with RPMI-1640 medium and then homogenizing

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clumps to obtain a single cell suspension. Bone marrow
cells were then centrifuged onto glass slides and
fluorescently stained as described in Example 8. This
experiment demonstrated that approximately 20% of mouse
5 bone marrow hematopoietic cells (including cells that on
the basis of morphology are primitive myeloblastic and
erythroblastic precursor cells) were transfected *in vivo*
by a single ip dose of pZN27:DDAB:DOPE complexes.

Example 10

10 Demonstration that Human CD4^{+} T Lymphocytes, Freshly
Isolated
from Normal Donors, are Transfected *in vitro*.

Buffy coat preparations were freshly isolated from
normal human donors by gradient centrifugation. The
15 cells were then panned using an anti-CD3 (Becton-
Dickinson, Mountain View, CA) monoclonal antibody to
isolate the CD3^{+} T lymphocyte fraction. These cells were
then transfected using the following protocol: 10 million
cells were plated on 100 mm dishes and then 25 μg of
20 pZN27 complexed to 50 nmoles of DDAB:DOPE (1:1) SUV lipid
carriers were added for 48 hours. Control cells were not
transfected. The cells were then incubated with a FITC-
conjugated monoclonal anti-CD4 antibody (Becton-
Dickinson) and sorted by FACS. The resulting CD4^{+} T
25 lymphocytes were cytospun onto microscope slides and
fixed and probed for the presence of intracellular CAT
protein after permeabilizing the cells using 0.25% Triton
x-100. The cells were incubated with an anti-CAT
monoclonal antibody for 1 hr at 20°C and then stained
30 with a Texas Red conjugated goat anti-mouse IgG for 1 hr
at 20°C.
Results:

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Results are shown in Figure 4 and demonstrate that
at least 70% of freshly isolated human CD4$^+$ T lymphocytes
are transfected after exposure to pZN27:DDAB:DOPE
complexes in culture as shown by the red fluorescence of
5 cells in 4B. Control untransfected cells showed no
fluorescence. These results suggest that this approach
may dramatically improve the therapy of diseases
including AIDS and cancer.

As the above results show, high level transgene
10 expression has been achieved in heart, kidney, lymph
nodes, bone marrow cells, liver, lung and spleen after
systemic (iv or ip) transgene administration.
Transfection of the heart, kidney, lymph nodes or bone
marrow cells individually after systemic (iv or ip)
15 transgene administration into adults has not previously
been accomplished. Transfection of T lymphocytes, lung
airway or alveolar cell types, cardiac endothelial lining
cells and cardiac muscle cells, and bone marrow
hematopoietic precursor cells *in vivo* by systemic
20 administration of DNA has not been shown previously.
Specifically, greater than 50% of T lymphocytes, lung
airway epithelial, alveolar and vascular endothelial cell
types, cardiac endothelial lining cells and bone marrow
hematopoietic precursor cells (including about 70% of
25 blast cells) are transfected *in vivo*, following one iv or
ip injection of CAT expression plasmid-cationic lipid
carrier complexes. Transfection of a high percentage of
all the cells present in any single tissue has not been
reported previously.

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Example 11

Efficient transfection of a variety of human lung cancer cell lines using cationic liposome-mediated delivery of DNA.

5   Method:

Cell Culture: NCI-H69, NCI-H82, and NCI-H520 cells were used. H69 and H520 cells were grown in RPMI-1640 with 10% fetal bovine serum (FBS) and H82 cells were grown in Dulbecco's minimum essential medium (DME)-H21 with 10%

10 FBS.

Liposome preparation: Liposomes were prepared as follows: a total of 4 μmoles of lipid dissolved in chloroform, (or in ethanol (DOTMA)) were evaporated to dryness on a rotary evaporator. One ml of 50 mM Tris,

15 0.5 mM EDTA, 50 mM NaCl, 100 μM $ZnCl_2$ buffer per 20 mmoles of lipid was added, and the mixture was sonicated in a bath sonicator (Laboratory Supply Co., Hicksville, NY) for 20 min. The resulting liposomes have an approximate mean diameter of 100 $\pm$ 25 nm. The following

20 liposome preparations were used: pure DOTMA, DOTMA:Cholesterol in a 2 to 1 molar ratio, pure L-PE or L-PE:CEBA in a 6 to 4 molar ratio.

Cellular transfection: For transfection of cells, 2 x 10^6 cells in 4 ml of serum-free medium were plated in 100

25 mm plastic petri dishes (Falcon, Oxnard, CA). The plasmid DNA-liposome complex was prepared by first adding 1) DNA and then 2), liposomes and mixing gently. The complex was then suspended in 1 ml of serum-free medium and added to the cells. Four hours later, the cells were

30 washed twice, resuspended in 10 ml of serum-containing medium, and subsequently harvested, 44 hours later. Just prior to harvesting, the cells were washed 2 times, and the plates were then scraped with a rubber policeman. The cells were centrifugated at 1,000 x g for 5 min, and

35 0.135 ml of 0.25 M Tris buffer pH 7.5, 5 mm EDTA was

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added to each pellet. The cells were freeze-thawed 3x,
heated at 65°C for 10 min, and spun at 12,500 x g for 10
min. The supernatant was assayed for protein and 20 μg
of supernatant protein per sample was used to measure CAT
5   activity, as described in Example 4.
Results: The results demonstrate the ability of cationic
liposomes to mediate high level transfection of two
different human small cell lung cancer lines (H69 and
H82) and a squamous cell lung cancer line (H520). All
10 three lines were very efficiently transfected by RSV-CAT
when complexed to 3 different cationic liposome
formulations (Figure 21). These human cell lines were
transfected either as, or more efficiently than rodent
tumor cell lines transfected under comparable conditions.

15   Example 12
        Transfection of lung cancers in mice by intravenous
            injection of cationic lipid carrier:DNA complexes

    Mouse: C57/black 6, female, 25 grams.
    Cancer: B16, mouse melanoma line which is highly
20   metastatic to lung. The cell line was grown
                   in RPMI 1640 medium supplemented with 5%
                   fetal calf serum.
    Lipid carrier: DOTAP:Cholesterol=1.1, 10mM in 5%
                   dextrose in water.
25 Plasmid: pZN20.
    Ratio: Cationic lipid:DNA=6 nmoles:1 μg, 100 μg in
                   200 μl of 5% dextrose in water were injected
                   by tail vein into each mouse.
    Inoculation of cancer cell line into mice and
30 administration of CAT expression plasmid-cationic lipid
    carrier complexes:
            B16 cells were trypsinized off the plates and
    50,000 cells were inoculated into each mouse by

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intravenous injection into the tail vein. Two weeks
after injection, cationic lipid carrier-DNA complexes
were injected via tail vein. Lungs were collected 48
hours postinjection, infused with 33% O.C.T., frozen in a
5 dry ice-ethanol bath, cryosectioned, and processed for
immunohistochemical analysis to detect intracellular CAT
protein.

Immunohistochemical analysis: Procedure:

        Organs were removed, appropriately trimmed,
10 embedded in OCT and snap frozen. Frozen tissues were
sectioned at 6 μm, collected onto glass slides, fixed for
10 minutes in 4°C acetone and then placed in 0.2% Triton
X-100 to permeabilize membranes. Sections were then
incubated for 12-48 hours with the monoclonal anti-CAT
15 antibody or isotype negative control antibody at the
appropriate dilution. After washing, a biotinylated
antibody directed against the primary antibody (Zymed, S.
San Francisco) was added for a minimum of 60 minutes,
followed by application of the streptavidin-peroxidase
20 complex (Zymed) for 60 minutes and then application of
the substrate-chromogen appropriate for the enzyme label
used. Slides can then be counter-stained in dilute
hematoxylin or left unstained and coverslipped in water-
soluble mounting media for examination.

25   Results:

        Immunohistochemical analysis are shown in Figure 8
and demonstrate that B-16 melanoma lung tumors (8A,
indicated by arrows) as well as intravascular tumor
emboli (8B, indicated by arrows) are efficiently
30 transfected after iv injection of DNA-lipid carrier
complexes. Both lung tumors and intravascular tumor
emboli show intense staining, indicating efficient, a
generalized transfection *in vivo*. Tumor bearing mice,
which did not receive an injection of DNA-lipid carrier
35 complexes, show no CAT activity in the lung or in any

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lung tumor cells (8C). The ability to transfect tumors present within mammalian hosts by systemic administration of a cloned gene has not previously been demonstrated.

Example 13
5   Demonstration of High Level CAT Gene Expression in Multiple
 Tissues After Intravenous (iv) Injection of pZN27 Alone, or
        pZN27:DDAB:Cholesterol SUV Complexes.

10 Lipid carrier: DDAB:Cholesterol=1:1, stock 10mM in 5%
                      dextrose. After addition of 5% dextrose
                      to the dried lipid film, the SUV were
                      prepared by sonication in a bath
                      sonicator for 20 minutes.

15 Plasmid: pZN27.
    DNA:Lipid carrier Ratio: Cationic lipid:plasmid DNA = 5
    nanomoles:1 μg DNA.
    DNA dose:
        pZN27 alone: Individual mice received 500 μg, 1
20 mg, 2 mg, or 500 μg, followed 4 hours later by a second
    500 μg dose, respectively of pZN27 in 200 μl 5% dextrose
    in water by tail vein injection.
        pZN27 complexed to lipid carriers: 100 μg plasmid
    DNA complexed to 500 nanomoles to DDAB:Cholesterol SUV
25 lipid carriers in 200 μl 5% dextrose in water was
    injected by tail vein per mouse.
    Mice: ICR, female, 25 grams.
    Tissue extraction procedure: Each organ was homogenized
    in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM EDTA, and the
30 resulting extract was centrifuged and the supernatant was
    then subjected to 3 cycles of freeze-thaw and then heated
    to 65°C for 20 min.

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<u>CAT assay procedure</u>: The protein concentration of each
tissue extract was quantitated using a Coomassie Blue-
based protein assay (BioRad, Richmond), and same amount
of total protein from each tissue extract was added in

5   the CAT assay, together with 10 μl of 20 mM acetyl CoA+12
μl of ^{14}C-chloramphenicol (25 μCi/ml, 55 mCi/mmole,
Amersham)), at 37°C for 13 hrs.
<u>Results</u>:
        Significant levels of CAT gene expression were

10 seen in each of the 6 different tissues assayed (lung,
heart, liver, kidney, spleen, and lymph nodes) after
injection of either pZN27 alone, or pZN27 complexed to
DDAB:Cholesterol lipid carriers. Expression of a
transgene in multiple tissues <i>in vivo</i> after systemic

15 injection of a naked expression plasmid previously has
not been demonstrated.

<u>Example 14</u>
<u>Induction of High Levels of Human Interleukin-2 in the</u>
<u>Spleen</u>

20 <u>and Lymph nodes of Mice by Intravenous Injection of</u>
<u>Cationic</u>
    <u>Lipid carriers Complexed to a CMV-Interleukin-2 Gene</u>

        <u>Mouse</u>:   C57/black 6, female, 25 grams.
        <u>Cancer</u>:   B16, mouse melanoma line which is highly

25   metastatic to lung. The cell line was
                    grown in RPMI-1640, 5% fetal calf serum.
        <u>Lipid carrier</u>: DDAB:Cholesterol=1.1, 10 mM in 5%
    dextrose in water.
        <u>Plasmid</u>: pZN46 (the HCMV promoter enhancer fused

30   to the human interleukin-2 coding
                    sequence).

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Ratio: Cationic lipid:DNA=5 nmoles:1 μg
                          DNA in 200 μl of 5% dextrose in
                          water administered per injection.

Inoculation of the tumor cell line into mice and
5 administration of a human interleukin-2 expression
plasmid-cationic lipid carrier complexes:

        B16 cells were trypsinized off the plates, and
50,000 cells were inoculated into each mouse by
intravenous injection into the tail vein. Starting 2
10 days after the tumor cell injection, cationic lipid
carrier-DNA complexes were injected via tail vein 2 times
per week for a total of 2 weeks. The animals were
sacrificed 2 weeks post tumor cell injection, the spleen
and lymph nodes were removed, rendered into single cell
15 suspensions using a tissue grinder, and then cultured for
24 hours in RPMI-1640, 10% fetal calf serum in 100 mm
plastic dishes in a 37°C incubator. After 24 hours the
supernatant was collected and the concentration of human
interleukin-2 in the supernatant was determined using a
20 human IL-2 ELISA.

Results:

        One hundred picograms of human IL-2/ml per ml was
present in the spleen cell supernatant and 91 pg/ml IL-2
was present in the lymph node cell supernatant from the
25 mouse which was injected with the pZN46-DDAB:Cholesterol
lipid carrier complexes. No human IL-2 was detected in
either spleen cell or lymph node cell supernatants
derived from mice which received an identical injection
of B-16 melanoma cells, but which did not receive the
30 pZN46-DDAB:Cholesterol lipid carrier complexes. Thus,
substituting the human IL-2 gene coding region for the
CAT gene coding region in a HCMV expression plasmid
resulted in high level expression of the IL-2 gene *in
vivo* and the production of large amounts of a secreted
35 human IL-2 protein in a mouse.

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Example 15

Induction of High Level Expression of the Human CFTR gene

in Mice Treated by iv Administration of pZN32:Cationic

5   Lipid carrier Complexes

Mice: ICR female, 25 grams

Lipid carrier: DDAB:Cholesterol=1:1 SUV, 10 mM in 5% dextrose in water

Plasmid: pZN32 (the HCMV promoter enhancer fused to the

10 human CFTR coding sequence)

Ratio: Cationic lipid:DNA=5 nmoles:1μg plasmid DNA.

Dose: A total of 100 μg of plasmid DNA in 200 μl of 5% dextrose in water administered per iv tail vein injection.

15 Procedure:

Forty-eight hours after injection the mice were sacrificed, the lungs were removed and trimmed, embedded in OCT, and snap frozen. Frozen tissues were sectioned at 6 μm, collected onto glass slides, and the fixed for

20 10 minutes in 4% acetone. Immmunolocalization of CFTR was then performed using the affinity purified rabbit polyclonal anti-CFTR antibody, α-1468 (Cohn, *et al.*, (1991) *Biochem. Biophys. Res. Comm.* 181:36-43). The procedure used was identical to the one described in

25 Marino *et al.*, (1991) *J. Clin. Invest.* 88:712 with the following variation. After washing, a biotinylated antibody directed against the rabbit polyclonal antibody (Zymed) was added for 60 minutes, followed by application of the streptavidin phosphatase complex (Zymed) for 60

30 minutes and then application of the substrate-chromogen. Slides were then coverslipped in water-soluble mounting media for examination.

Results:

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Photomicrographs of frozen sections (viewed at different magnifications) of mouse lung 48 hours following iv injection to pZN32-DDAB:Cholesterol (1:1) liposome complexes and lung from untreated control are

5 shown in Figures 12A-E. As demonstrated by the intense staining with the polyclonal anti-CFTR antibody, α-1468, the overwhelming majority of the airways were transfected with the human CFTR gene. In 12A, 12C and 12E. by visual inspection, essentially all the cells in

10 transfected airways stain positively, demonstrating that the overwhelming majority of airway cells are transfected with the human CFTR gene in vivo with a single iv dose of pZN32 complexed to DDAB-cholesterol (1:1) liposome- treated and control animals could not be distinguished

15 histologically. Significant expression of the human CFTR gene is present in at least 50% of all the airways and at least 50% of all of the airway lining cells (by visual inspection) in mouse lungs for at least 60 days following a single iv dose of pZN32 complexed to DDAB-Cholesterol

20 (1:1) liposomes. Frozen sections of mouse lungs from control animals (12B and 12D) do not show any detectable staining for CFTR, confirming that all the CFTR expression present in 12A, 12C and 12E is due to transfection of lung cells with the human CFTR gene.

25   As shown by the above results, a single iv dose of an expression construct, containing a gene of interest, complexed to cationic liposomes transfects the majority of the cells lining the conducting airways of the lung, the gene product is present in the lung for at least 60

30 days, the expression appears to be airway cell-specific, and there is no histological evidence of damage following exposure. This is important because liposomes are well tolerated and non-immunogenic. Furthermore, the appearance, behavior, and life span of mice treated with

35 intravenously injected pZN32:DDAB-Cholesterol (1:1)

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complexed appear normal, and are indistinguishable from
untreated, normal control animals. This lack of
toxicity, as demonstrated by analysis of appearance,
behavior, life span and detailed histologic analysis of a
5   wide variety of tissues from these animals demonstrates
the lack of toxicity produced by *in vivo* delivery of
pZN32-DDAB:Cholesterol complexes. Additionally, the
effects of repeated iv administration of the DNA/liposome
complexes is effective and is non-toxic. The cationic
10 liposome-mediated DNA delivery by iv injection provides
high level, lung-specific transgene expression *in vivo.*

Example 16

Demonstration of CAT Gene Expression in Lung and Liver
After Intravenous Injection of Different CAT gene-
15 Containing Plasmids

Lipid carrier: DDAB:Cholesterol= 1:1, stock 5mM in 5%
                    dextrose in water.
Plasmids: Plasmids are indicated below.
DNA-Lipid carrier Ratio: Cationic lipid: plasmid DNA = 1
20 nanomole: 1 μg
Dose: 100 μl DNA in 200 μl volume injected
              intravenously by tail vein injection.
Mice: ICR, female, 25 g
Procedure: The animals were sacrificed 24 hours after
25 injection. The tissue extraction procedure and CAT assay
were as described in Example 4 except that the CAT assay
was incubated for 3 hr at 37°C and 2.0 mM paraoxon (Lai,
et al., (1988) *Carcinogenesis* 9:1295-1302) was added to
the liver samples. The results are shown in Figure 15.
30 Lanes 1-12 are lung samples, lanes 13-24 are liver
samples. Lanes 1, 2, 13, 14 are pZN51; lanes 3, 4, 15,
16 are pZN60; lanes 5, 6, 17, 18 are pZN61; lanes 7, 8,

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19, 20 are pZN62; lanes 9, 10, 21, 22 are pZN63; and
lanes 11, 12, 23, 24 are pZN27.

Results:

      pZN51, which does not contain an intron, is
5 expressed as well as, or better than, plasmids containing
an intron either 3' or 5' to the coding sequence.

Example 17
Generalized versus tissue and cell type-specific CAT gene expression produced by iv injection of CMV-CAT-liposome
10   or CFTR-CAT-liposome complexes, respectively.

Mouse: ICR female, 25 grams.

Liposome: DDAB:Cholesterol=1:1 SUV, 10mM in 5% dextrose
           in water.

Plasmid: 1) pZN27 or 2) pBE3.8CAT (see Chou et al.,
15   (1991) J. Biol Chem 266:24471-24476, for
        construction).

Experimental conditions: Mice in groups of 3 received
either (a), no treatment, or (b), a single iv tail vein
injection of DDAB:Cholesterol liposomes complexed to 100
20 µg of) a 3.8 kb sequence of the 5' upstream region of the
human CFTR gene fused to the CAT gene (pBE3.8CAT) or (c)
pZN27. Mice were sacrificed 24 hours later and CAT
activity assayed in lung, liver, spleen, lymph nodes,
kidney and heart, as described in Example 4.
25 Immunohistochemical analysis of lung section from each of
the groups was performed as described in Example 3.

Results:

      Immunohistochemical staining of frozen lung
sections from these mice showed that iv injection of CMV-
30 CAT-liposome complexes produced high levels of red
staining, indicating CAT gene expression in endothelial,
alveolar and airway cells within the lung (16A). In
contrast, CFTR-CAT-liposome complexes produced CAT gene

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expression primarily localized in airway epithelial cells
(16B). This approximates the pattern of endogenous CFTR
gene expression in rat lung, as determined by *in situ*
hybridization studies (Trezise and Buchwald, (1991)
5   *Nature* 353:434-437. A lung section from an uninjected
mouse does not show red staining, indicating that CAT
gene expression is present only in transfected cells
(Fig. 16C). Figure 16D is a high magnification
photomicrograph of alveoli from a CMV-CAT treated mouse
10 and shows a high level of CAT gene expression in both
alveolar cells and lung endothelial cells. A high
magnification photomicrograph of alveoli from CFTR-CAT
treated mice (Figure 16E) shows no significant CAT gene
expression in either alveolar or endothelial cells,
15 demonstrating that the CFTR promoter targets transgene
expression to airway epithelial cells. This is the first
demonstration that transgenes can be expressed within
mouse lung in either a generalized or cell type-specific
fashion depending on the regulatory element used, after
20 iv injection.

        CAT assay demonstrated that CMV-CAT produced
significant CAT gene expression in the lung, liver,
heart, spleen, lymph nodes and kidney, whereas CFTR-CAT
produced lung-specific gene expression. Photographs of
25 autoradiographic analysis of each tissue are shown in
Figure 16 (F-K). Thus, the CMV promoter induces
expression of a linked gene in a wide range of tissues,
whereas the 5' flanking region of the human CFTR gene
directs tissue-specific transgene expression after iv,
30 liposome-based administration.

                    Example 18
    Comparison of transfection focusing on iv injection of
plasmid

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alone with iv injection of plasmid complexed to a lipid
carrier.

Demonstration of widespread, high level CAT gene
expression in in vivo after intravenous (iv) injection of
5 pZN27 alone.
          Plasmid: pZN27.
          DNA:Liposome Ratio: Plasmid DNA alone, without
liposomes, was injected.
          DNA dose: 1 mg plasmid DNA in 200 μl 5% dextrose
10 in water was injected times 2 over a 4 hour period by
tail vein per mouse.   Mice were sacrificed 24 hours later
and 17 different tissues were assayed for CAT gene
activity.
          Mice: ICR, female, 25 grams.
15   Tissue extraction procedure: Each organ was
homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM
EDTA, and the resulting extract was centrifuged and then
subjected to 3 cycles of freeze-thaw and then treated to
65°C for 20 min.
20   CAT assay procedure: The protein concentration of
each tissue extract was quantitated using a ninhydrin-
based protein assay (BioRad, Richmond), and the same
amount of total protein from each tissue extract was
added in the CAT assay, together with 10 μl of 20 mM
25 acetyl CoA+12 μl of ^{14}C-chloramphenicol (25 μCi/ml, 55
mCi/mmole, Amersham), at 37°C for 13 hrs.
          Results: An autoradiograph of this experiment is
shown in 19A.   Compared to control levels (lane 1), iv
injection of pZN27 alone produced highly significant
30 levels of CAT gene expression in the following tissues:
lung, thymus, esophagus, heart, liver, spleen, stomach,
small intestine, caecum, ovary, vagina, skeletal muscle,
pancreas and lymph nodes.   Thus, iv injection of the

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pZN27 expression plasmid alone can efficiently transfect
a very large and diverse number of tissues in the body.

<u>Demonstration of widespread, high level CAT gene</u>
<u>expression <i>in vivo</i> after intravenous (iv) injection of</u>
5 <u>pZN27 complexed to DDAB:Cholesterol (1:1) liposomes</u>.
        <u>Plasmid</u>: pZN27.
        <u>Liposome</u>: DDAB:Cholesterol=1:1, stock 10 mM in 5%
dextrose.
        <u>DNA:Liposome Ratio</u>: liposome:plasmid 5 nmoles:1
10 μg
        <u>DNA dose</u>: 100 μg plasmid DNA in 200 μl 5%
dextrose in water was injected by tail vein per mouse.
Mice were sacrificed 24 hours later and 17 different
tissues were assayed for CAT gene activity.
15   <u>Mice</u>: ICR, female, 25 grams.
        <u>Tissue extraction procedure</u>: Each organ was
homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM
EDTA, and the resulting extract was centrifuged and then
subjected to 3 cycles of freeze-thaw and then treated to
20 65°C for 20 min.
        <u>CAT assay procedure</u>: The protein concentration of
each tissue extract was quantitated using a ninhydrin-
based protein assay (BioRad, Richmond), and same amount
of total protein from each tissue extract was added in
25 the CAT assay, together with 10 μl of 20 mM acetyl CoA+12
μl of ^{14}C-chloramphenicol (25 μCi/ml, 55 mCi/mmole,
Amersham), at 37°C for 13 hrs.
        <u>Results</u>: An autoradiograph of this experiment is
presented in Figure 19B. Compared to control levels
30 (lane 1), iv injection of pZN27:DDAB:Cholesterol
complexes produced high levels of CAT gene expression in
the following tissues: lung, thymus, esophagus, heart,
liver, spleen, stomach, small intestine, large intestine,
caecum, uterus, ovary, vagina, skeletal muscle, pancreas

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and lymph nodes. Thus, iv injection of
pZN27:DDAB:Cholesterol complexes can efficiently
transfect a very large and diverse number of tissues in
the body.

5   Example 19
    IV Injection Of GM-CSF Expression Plasmid-Cationic
Liposome
    Complexes Produces Significant Antitumor Effects.

        Mouse: C57/black 6, female, 25 grams.
10   Cancer: B16, mouse melanoma line which is highly
                   metastatic to lung. The cell line was
                   grown in RPMI-1640, 5% fetal calf serum.
        Liposome: DDAB:Cholesterol=1:1, 10 mM in 5%
                   dextrose in water. Twenty five thousand
15   B-16 cells were injected iv by tail
                   vein.
        Plasmid: pZN84 (the HCMV promoter enhancer fused
                   to the murine GM-CSF coding sequence as
                   shown in Figure 17).
20   Ratio: Cationic lipid:DNA=5 nmoles:1 μg.
                       A total of 100 μg of plasmid DNA in
                       200 μl of 5% dextrose in water
                       administered per injection.
    Experimental outline: Mice in groups of eight received a
25 single iv tail vein injection of 2.5 x 10^4 B-16 melanoma
    cells. Group 1 received no treatment, group 2 received
    bi-weekly injections of 100 μg of pZN84 complexed to
    DDAB:Cholesterol liposomes, beginning 4 days prior to
    tumor cell injection and continuing for 2 weeks following
30 tumor injection. All mice were sacrificed 3 weeks
    following tumor cell injection and surface lung tumor
    nodules which were black and macroscopic were counted
    using a dissecting microscope.

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Results:

Control animals had 64.5 ± 19.7 (S.E.M.) nodules
per lung. In contrast, the GM-CSF-liposome-treated
animals had 11.9 ± 3.6 tumor nodules per lung (p < 0.01
5 versus the control group, as assessed by Student's t
test). Thus, iv injection of a cytokine gene produced a
highly significant antitumor effect. This is the first
demonstration that iv injection of a gene can produce
anti-tumor activity in vivo.

10   Example 20
     Prolonged, high level murine GM-CSF gene expression in
vivo
     after intravenous (iv) injection of pZN84 complexed to
          DDAB:Cholesterol (1:1) liposomes into goats.
15 Animals: Goats: female, 110 pounds
    Plasmid: pZN84
    Liposome: DDAB:Cholesterol=1:1, stock 10mM in 5%
    dextrose
    DNA:Liposome Ratio: Liposome:plasmid=5:1
20 DNA dose: 1.0 mg plasmid DNA in 5 ml 5% dextrose in
    water was injected by jugular vein per goat.
    Blood Sampling: Blood was drawn just prior to injection
    of pZN84 liposome complexes, and at 12, 24, 48, 72, 168
    and 840 hours post-injection.
25 Murine GM-CSF ELISA assay procedure: Murine GM-CSF was
    measured in these serum samples using a commercial murine
    GM-CSF ELISA kit provided by Endogen. There is no cross
    reactivity between goat and murine GM-CSF by ELISA.
    Results: As shown in Figures 22 and 23 (Goats A, B and
30 C), as compared to control levels, iv injection of
    pZN84:DDAB:Cholesterol complexes produced high sustained
    circulating levels of mouse GM-CSF protein for at least
    168 hours following injection. However, by 840 hrs (35
    days) GM-CSF levels were substantially reduced (Figure

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27, Goat A). No mouse GM-CSF protein was detected in the
circulation of a goat which received 1 mg of the CAT gene
(pZN51) complexed to DDAB:Cholesterol liposomes by the
identical iv injection protocol. The goat receiving the

5 CAT gene did express CAT antigen on the majority of its
circulating white blood cells at 24 hours after
injection, as determined by immunostaining for CAT
antigen. No CAT antigen was detected in the white blood
cells of the goats which were injected with GM-CSF gene-

10 liposome complexes. Thus, iv injection of
pZN84:DDAB:Cholesterol complexes can produce high level
expression of the murine GM-CSF gene for prolonged
periods in goats. IV injection of pZN51:DDAB:Cholesterol
complexes can produce high levels of the CAT gene product

15 in the majority of all circulating white blood cells in
goats.

Example 21
Influence of liposome-GM-CSF plasmid complex on the
course of

20   experimental viral pneumonia in mice.

Endogenous cytokines are believed to be included
in the host response to a viral infection. It is
therefore of interest to determine whether increased
expression of a single cytokine, GM-CSF would influence

25 the time course of viral pneumonia. The effect of
delivering the murine GM-CSF plasmid-liposome complex
via two different routes on the host response to a Sendai
virus challenge is tested. The plasmid-liposome complex
is delivered iv to one group of mice, and intranasally

30 (in) to a second group. Dose, time of delivery, virus
challenge dose and endpoints are the same for both
groups. Dose of plasmid-liposome complex is 100

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μg/mouse/dose. Dosing for both protocols occurs on the
following scale:

Group	Initial PL* dose	2nd PL dose	Virus
challenge	Endpoints		
IV Route	day-3	day 0	day 0
days 3, 7, 10			
IN Route	day-3	day 0	day 0
days 3, 7, 10			

*PL - plasmid-liposome

Each experimental group consists of the following:

murine GM-CSF plasmid-liposome + virus: 3
mice

CAT plasmid-liposome + virus: 3 mice

Untreated + virus: 3 mice

This results in 9 mice per timepoint (27 total for
each experiment, 54 total mice). All animal procedures
are performed via NIH and AAAI.AC guidelines including
use of appropriate analgesia.

Virus challenge is administered intranasally with
Sendai virus strain 771076, a strain known to be
pathogenic in mice. Endpoints include the following
(these have been shown to useful endpoints for Sendai
pneumonia):

1. lung weight vs. body weight and brain weight
2. lung histopathology
3. quantitative virus recovery
4. Sendai specific antibody response
5. lung GM-CSF
6. lung TNF

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Example 22
High level expression of the CAT gene in mouse brain produced by injection
of DNA alone or DNA-cationic liposome complexes directly into the

central nervous system.

Mice: ICR, female, 25 grams

Plasmid: pCIS-CAT

Liposome: DOTMA:DOPE (1:1)

For each mouse injected, 2.5 μg DNA was diluted in 5% dextrose, then mixed with liposomes diluted to the same volume in 5% dextrose. Five microliters were injected stereotactically into the right ventricle of each mouse.

Ratio: Plasmid:lipid = 1:0 (1 mg DNA: 0 μmoles DOTMA)

Plasmid:lipid = 1:1 (1 mg DNA: 1 μmoles DOTMA)

Plasmid:lipid = 1:3 (1 mg DNA: 3 μmoles DOTMA)

Plasmid:lipid = 1:4 (1 mg DNA: 4 μmoles DOTMA)

Plasmid:lipid = 1:6 (1 mg DNA: 6 μmoles DOTMA)

Mice were sacrificed 48 hr. post injection. Brains were removed and separated into left and right hemispheres. Each hemisphere was homogenized in 250 μl 0.25 M Tris pH 7.8, 5 mM EDTA followed by three cycles of freeze-thaw then 10 min. at 65°C.

The amounts of extracts used for CAT assay were normalized to protein levels. 0.3 ml of ^{14}C-chloramphenicol was used for each assay. Assays were carried out at 37°C overnight. The reaction products were separated on TLC plate and exposed to film, as described in Example 4.

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Results: These results demonstrate that high
level expression of a heterologous gene can be produced
throughout the brain by injection of DNA-liposome
complexes (at appropriate ratios) directly into the
5 central nervous system. They also demonstrate that
injection of an expression plasmid alone can produce
significant transgene expression in the brain. The
results are shown in Figure 20.

Example 23
10 Demonstration of CAT gene expression in the lung after
intravenous (iv) injection of pRSV-CAT:L-PE:CEBA
complexes.

Lipid carrier: L-PE:CEBA=1:1, stock 20 mM in
lipid carrier buffer.
15   Plasmid: pRSV-CAT.
DNA:Lipid carrier Ratio: Lipid carrier:plasmid=1
nanomole cationic lipid: 1 μg plasmid DNA.
DNA dose: 100 μg plasmid DNA in 200 μl 5%
dextrose in water was injected by tail vein per mouse.
20   Mice: Balb/c, female, 25 grams.
Tissue extraction procedure: Forty eight hours
after tail vein injection, the animals were sacrificed,
whole lung was homogenized in 1ml of 0.25M Tris-HCl pH
7.8, 5mM EDTA, 80 μg/ml PMSF and the resulting extract
25 was centrifuged and then the supernatant was subjected to
3 cycles of freeze-thaw and then heated to 65°C for 20
min..
CAT assay procedure: 100 μl of extract+10 μl of
20 mM acetyl CoA+4 μl of ^{14}C-chloramphenicol (25 μCi/ml,
30 55 mCi/mmole, Amersham) were incubated together at 37°C
for 6 hr. At 3 hours, an additional 10 μl of acetyl CoA
was added.
Results:

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This experiment showed that a significant level of CAT activity (indicative of expression of the transgene) was present in the lung of the animal injected with lipid carrier:DNA complexes, but not present in the lungs from
5 control animals.

Example 24

Demonstration of CAT gene expression in multiple tissues
after intravenous (iv) injection of pZN20-CAT:DDAB:DOPE
complexes.

10   Lipid carrier: DDAB:DOPE=1:1, stock 10mM in 5%
dextrose.
         Plasmid: pZN20.
         DNA:Lipid carrier Ratio: Lipid
carrier:plasmid=(A) 3 nmole cationic lipid:
15   1 μg plasmid DNA (SUV). (B) 6 nmole cationic
lipid: 1 μg plasmid DNA (MLV).
         DNA dose: 100 μg plasmid DNA in 200 μl 5%
dextrose in water was injected by tail vein per mouse.
Three mice each received this dose of MLV:pZN20 and 3
20 mice each this dose of SUV:pZN20.
         Tissue extraction procedure: Each organ was
homogenized in 0.3 ml of 0.25 M Tris-HCl pH 7.8, 5 mM
EDTA, and the resulting extract was centrifuged and then
the supernatant was subjected to 3 cycles of freeze-thaw
25 and then heated to 65°C for 20 min..
         CAT assay procedure: The protein concentration of
each tissue extract was quantitated using a Coomassie
Blue-based protein assay (BioRad, Richmond, CA), and the
same amount of total protein from each tissue extract was
30 added in the CAT assay, together with 10 μl of 20 mM
acetyl CoA+12μl of ^{14}C-chloramphenicol (25 μCi/ml, 55
mCi/mmole, Amersham), at 37°C for 13 hrs.

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Results:

This experiment demonstrated that iv injection of pZN20:DDAB:DOPE complexes gave significant levels of CAT gene expression in each of 6 different tissues including

5   lung, heart, liver, spleen, kidney and lymph nodes. Furthermore, MLV lipid carriers mediated equal or higher levels of *in vivo* transgene expression than did SUV lipid carriers composed of the same lipids.

Example 25

10   Production of Vaccines

The subject invention may find general utility in the production of vaccine, *in vivo*, for a variety of diseases. It has been shown that chimeric, genetically-engineered proteins of antigen fragments linked to

15   granulocyte-macrophage colony-stimulating factor (GM-CSF), augment antigen presentation and increase immune response to the antigen when injected *in vivo* (Tao and Levy, (1993) *Nature* 362:755-758. Thus a nucleic acid sequence coding for a particular antigen may be fused to

20   the nucleic acid coding for GM-CSF and expressed in appropriate cells *in vivo* using this invention. This approach could dramatically improve the control of proliferation of malignant cells in the treatment of cancer. Other uses would include *in vivo* vaccine

25   production against a variety of viral and other infectious diseases, particularly those diseases in which an immune response has been unsuccessful or weak by conventional vaccine strategies. Of particular interest would be those diseases, such as HIV, which are

30   characterized by proteins with hypervariable domains. Nucleic acid sequences encoding a variety of possible hypervariable domain sequences could be fused to GM-CSF and expressed simultaneously *in vivo* to elicit an immune response against many different strains of the virus.

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Example 26
Treatment of Tumor Proliferation

Nucleic acid sequences such as those coding for protein 53 may be useful in treating tumor proliferation.
5 Sequences coding for the tumor suppressor protein p53 may be expressed intracellularly *in vivo* using appropriate expression vector-lipid complexes. Such complexes can be conveniently targeted to tumor cells by appropriate antibodies or ligands conjugated to the lipid complexes.
10 Initial information regarding transfection of cells is developed using cultured cells, such as the tumor cell line K562. Once p53 gene expression is verified in K562 cells and the optimal vector have been identified, the following *in vivo* anti-tumor experiment is conducted.
15 SCID mice receive approximately 25 million K562 cells per mouse by intraperitoneal injection. Approximately three (3) weeks after injection, tumor-bearing mice in groups of ten (10) receive the following: no treatment/control group; p53 vector liposome complexes by intravenous
20 injection; p53 vector liposome complexes by intraperitoneal injection; reported gene vector liposome complexes. The anti-tumor effects of the expression of p53 is then be assessed based upon survival of the mice.

Example 27

25 Erythropoiesis, the production of red blood cells, can cause continuously throughout the life span to offset cell destruction. Erythropoiesis is a very precisely controlled physiological mechanism and having sufficient numbers of red blood cells to be available in the blood
30 for proper tissue oxygenation, but not so many that the cells would impede circulation. The formation of red blood cells occurs in the bone marrow and is under the control of the hormone erythropoietin. Because of erythropoietin is essential in the process of red blood

cells formation, the hormone has potential use for
application in both the diagnosis of the treatment of
blood disorders characterized by low or defective red
blood cell production. Gene therapy using the coding
5   sequence for erythropoietin does find use in a variety of
disease states, disorders and states of hematologic
irregularity including anemia, in particularly correction
of anemia of a type associated with chronic renal failure
and the like. A nucleic acid sequence coding for a
10 polypeptide having EPO activity is inserted into an
appropriate transcription or expression cassette and
introduced into a host mammal as naked DNA or complexed
with an appropriate liquid carrier. Monitoring of the
production of active EPO polypeptide can be performed as
15 described for example in USPN 4,703,008.

As shown by the above results, a plurality of
tissues can be transformed following systemic
administration of transgenes, either complexed to a lipid
carrier or as naked nucleic acid. Expression of
20 exogenous DNA following intravenous injection of a
cationic lipid carrier/exogenous DNA complex into a
mammalian host has been shown in multiple tissues,
including T lymphocytes, metastatic tumors and
intravascular tumor emboli. Expression of exogenous DNA
25 in multiple different tissues, including those of the
reticuloendothelial system has been obtained following
intravenous injection of an expression plasmid as naked
DNA. The ability to transfect T lymphocytes *in vivo* will
have a dramatic impact on the treatment of AIDS, cancer,
30 multiple sclerosis, and arthritis. *In vivo* transfection
of cardiac endothelial cells will have a dramatic impact
on the treatment of heart disease and heart attacks. *In
vivo* transfection of lung cells will have a dramatic
impact on the treatment of cystic fibrosis, asthma,
35 emphysema and lung cancer. *In vivo* transfection of bone

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marrow cells will have a dramatic impact on the treatment
of cancer, blood diseases and infections.

The *in vivo* gene therapy delivery technology as
described above is non-toxic in animals and transgene
5   expression has been shown to last for at least 60 days
after a single administration. The transgene does not
appear to integrate into host cell DNA at detectable
levels *in vivo* as measured by Southern analysis,
suggesting that this technique for gene therapy will not
10   cause problems for the host mammal by altering the
expression of normal cellular genes activating cancer-
causing oncogenes, or turning off cancer-preventing tumor
suppressor genes. Additionally, transgene expression
after systemic administration of a DNA expression vector
15   alone has been shown; transgene expression was produced
in the lung for at least three weeks after a single
administration of a DNA expression vector without a
carrier system.

Systematic injection of heterologous genes into
20   adult animals can produce very high level transgene
expression in a wide range of tissues, and transfect the
majority (>70%) of all cells present in many of these
tissues as demonstrated above. In contrast, previous
studies attempting direct transfer of heterologous genes
25   into adult animals have reported transfection limited to
one or a few tissues, low levels of transgene expression
in these tissues and (whenever histochemical analysis was
included) transfection limited to less than 1% of the
cells present in transfected tissues.

30   In addition to transfecting the majority of all
cells present in the lung, using the methods and
constructs described above, high level transgene
expression has been obtained in a wide variety of other
tissues and cell types. These include:

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- Transfection of the majority of all cells
     present in the spleen and lymph nodes,
     including transfection of greater than 70% of
     all the T lymphocytes. The ability to
5   efficiently transfect T lymphocytes *in vivo*
     permitting for the first time specific
     molecular approaches to both anti-HIV therapy
     and to selective modulation of the immune
     response.
10 - Efficient transfection of visceral tumors and
     of intravascular tumor emboli after iv
     injection of DNA into tumor-bearing hosts.
     Previously, gene transfer studies involving
     cancer have been restricted solely to *ex vivo*
15   approaches. Our work now permits direct
     transfection with transgenes which provide
     tumor suppressor, anti-oncogene and/or anti-
     metastases activity within tumors in tumor-
     bearing hosts.
20 - Transfection of the majority of cardiac
     vascular endothelial cells, as well as bone
     marrow-derived hematopoietic cells, including
     the great majority of blast cells present in
     the bone marrow, by systemic delivery of
25   heterologous genes. These results create
     dramatic and new means for controlling
     ischemic heart disease and hematopoiesis at
     the molecular level.
   - Demonstration of the ability to produce high
30   level *in vivo* expression of a variety of
     biologically important transgenes, as
     exemplified by human CFTR, IL-2 and GM-CSF.
       All publications and patent applications are
   herein incorporated by reference to the same extent as if
35 each individual publication or patent application was

specifically and individually indicated to be
incorporated by reference.

        The invention now being fully described, it will
be apparent to one of ordinary skill in the art that many
5 changes and modifications can be made thereto without
departing from the spirit or scope of the appended
claims.

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<u>WHAT IS CLAIMED IS</u>:

     1.   A complex of an expression cassette comprising:

     as operably joined components, a transcriptional
5 and translational initiation regulatory region, a nucleic
acid sequence of interest, and a transcriptional
termination regulatory region, wherein said regulatory
regions are functional in the cells of a mammalian host,
and optionally, a cationic lipid carrier, and wherein
10 said nucleic acid sequence of interest is free of
introns.

     2.   A complex according to claim 1 wherein an
intron is positioned 5' to said nucleic acid sequence.

     3.   A complex according to claim 1 or 2 wherein
15 5' to said transcription and translational initiation
region is at least one of an enhancer sequence and an
intron.

     4.   A complex according to any one of the
preceding claims wherein said transcriptional initiation
20 regulatory region provides for tissue specific
expression.

     5.   A complex according to any one of the
preceding claims wherein said cationic lipid carrier is
prepared from one or more lipids selected from the groups
25 consisting of DDAB, DOTMA, DOPE, Cholesterol, and L-PE,
DOTAP, and CEBA.

     6.   A complex according to any one of the
preceding claims wherein said nucleic acid sequence of
interest is CFTR, or interleukin 2.

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7.   A complex according to claim 1 wherein the expression cassette is pCIS-CAT, pZN27, pZN51, pZN32, or pZN46.

8.   A complex of a transcription cassette
5   comprising: as operably joined components, a transcriptional initiation region, a nucleic acid sequence of interest, and a transcriptional termination regulatory region, wherein said regulatory regions are functional in the cells of a mammalian host, and
10   optionally a cationic lipid carrier.

9.   A complex according to claim 8, wherein said nucleic acid sequence of interest is an antisense sequence.

10.   A pharmaceutical composition comprising:
15   a complex according to any one of the preceding claims.

11.   A complex according to any one of claims 1 to 9 or a composition according to claim 10 for use in a method of treatment of the human or animal body.

20   12.   A complex or composition for use according to claim 11 wherein the method of treatment comprises the systemic introduction into the body of the complex or composition.

13.   Use of an expression cassette as defined in
25   any one of claims 1 to 7 or a transcription cassette according to any one of claims 8-9 for the manufacture of a medicament for the treatment of the human or animal body by systemic gene therapy.

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14. Use of a complex as defined in any one of claims 1 to 9 for the manufacture of a medicament for the treatment of the human or animal body by systemic gene therapy.

5   15. A non-human transgenic mammal wherein at least one cell type or tissue in addition to lung or liver contains a recombinant gene sequence introduced systemically into said mammal or an ancestor of said mammal.

10   16. A non-human transgenic mammal according to claim 15 wherein the recombinant gene sequence comprises an expression cassette as defined in any one of claims 1 to 7, or a transcription cassette as defined in any one of claims 8-9.

15   17. A non-human transgenic mammal according to claim 15 or 16 wherein said cell type or tissue is one or more cell type or tissue selected from the group consisting of spleen, lymph node, heart, kidney, bone marrow, and T-lymphocytes.
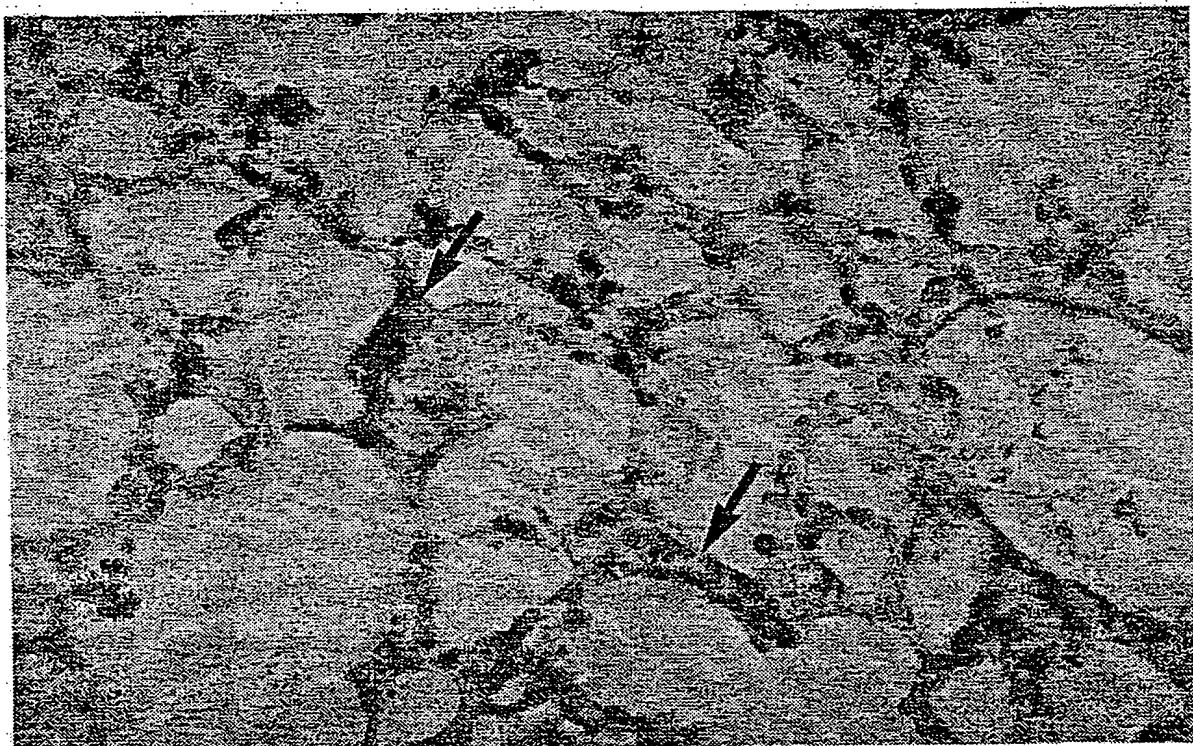
FIGURE 1A
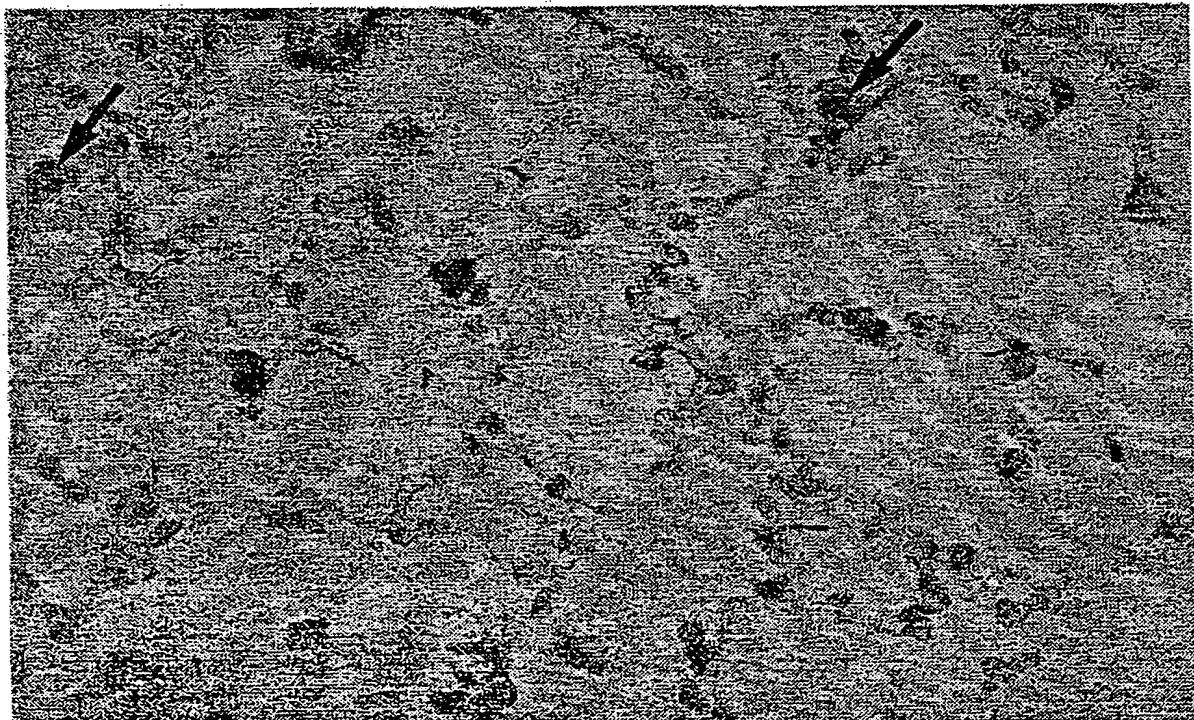
FIGURE 1B   Control
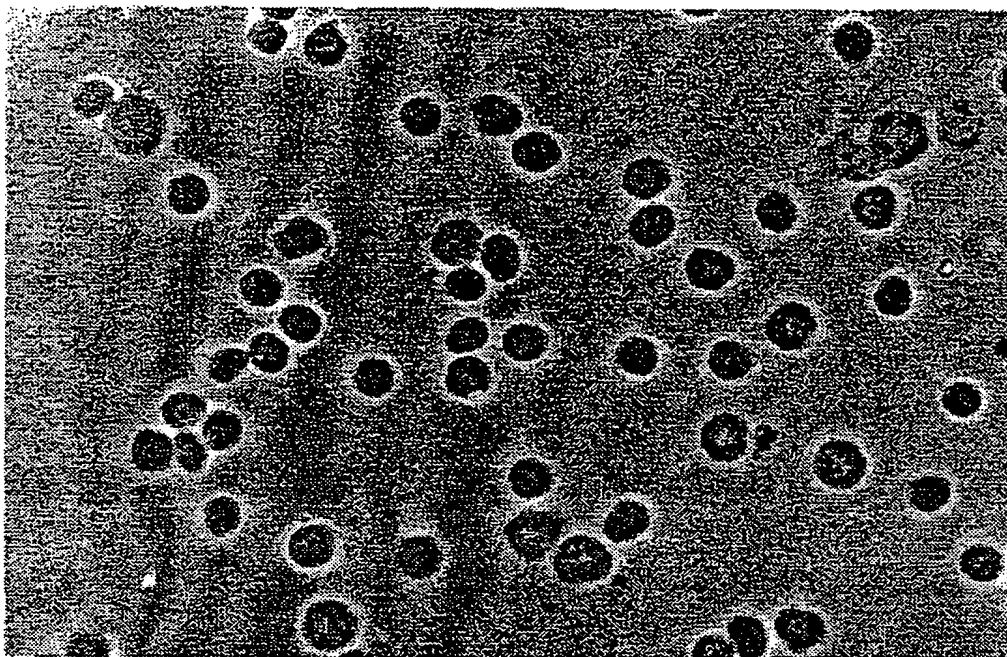
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Phase Contrast   FIGURE 2A
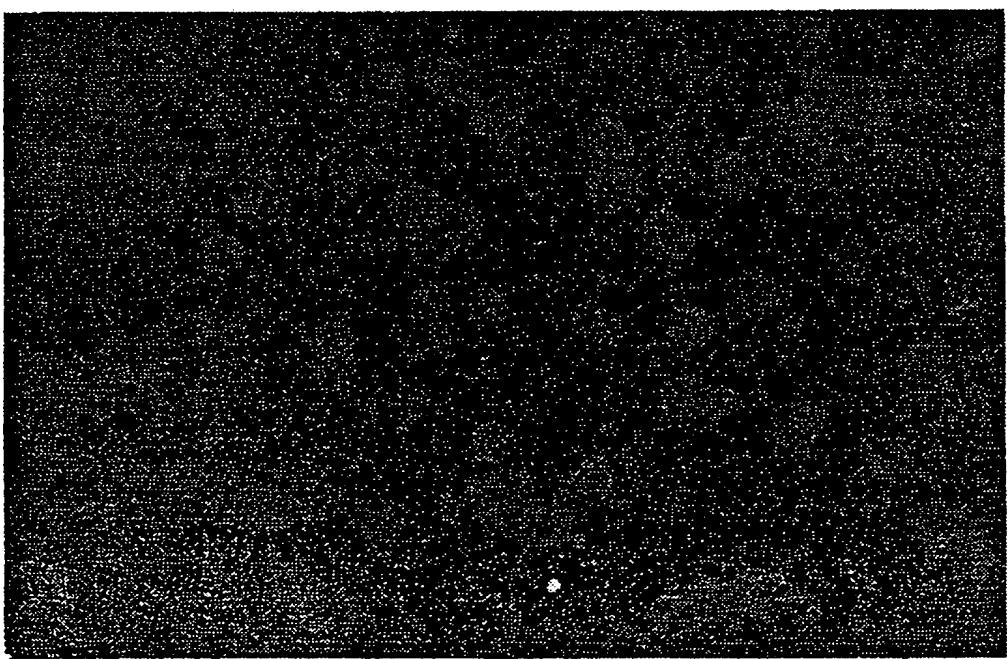
Fluorescent   FIGURE 2B
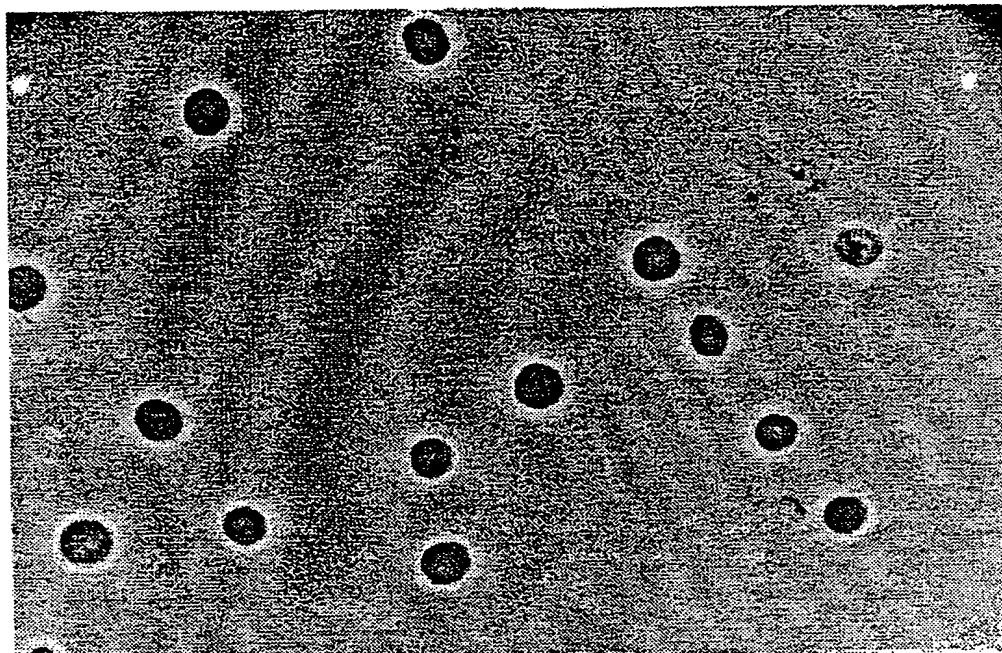
Phase Contrast   FIGURE 2C   Control
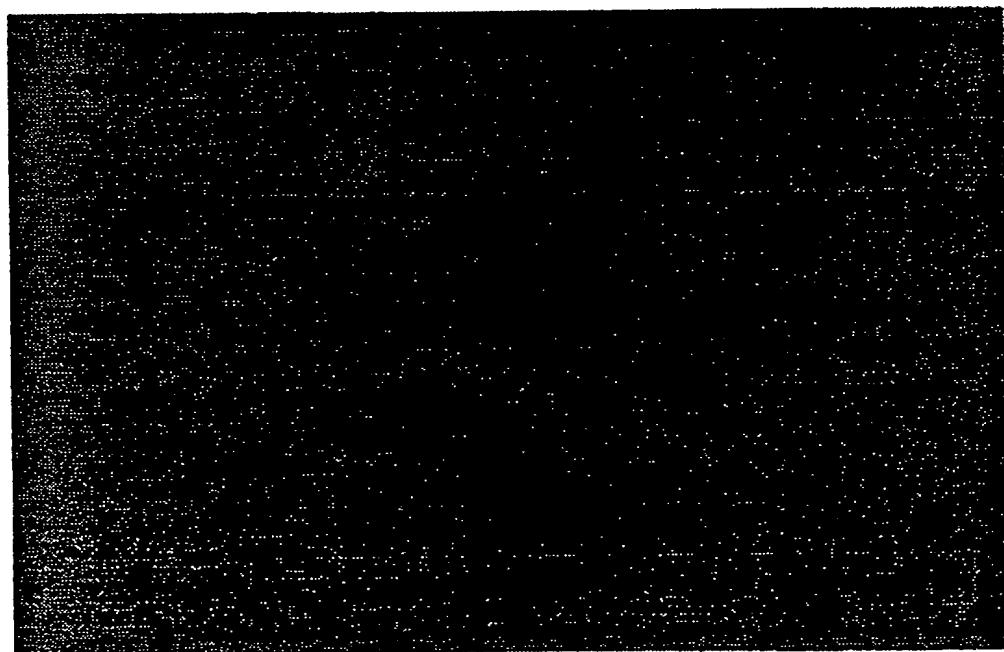
Fluorescent   FIGURE 2D   Control
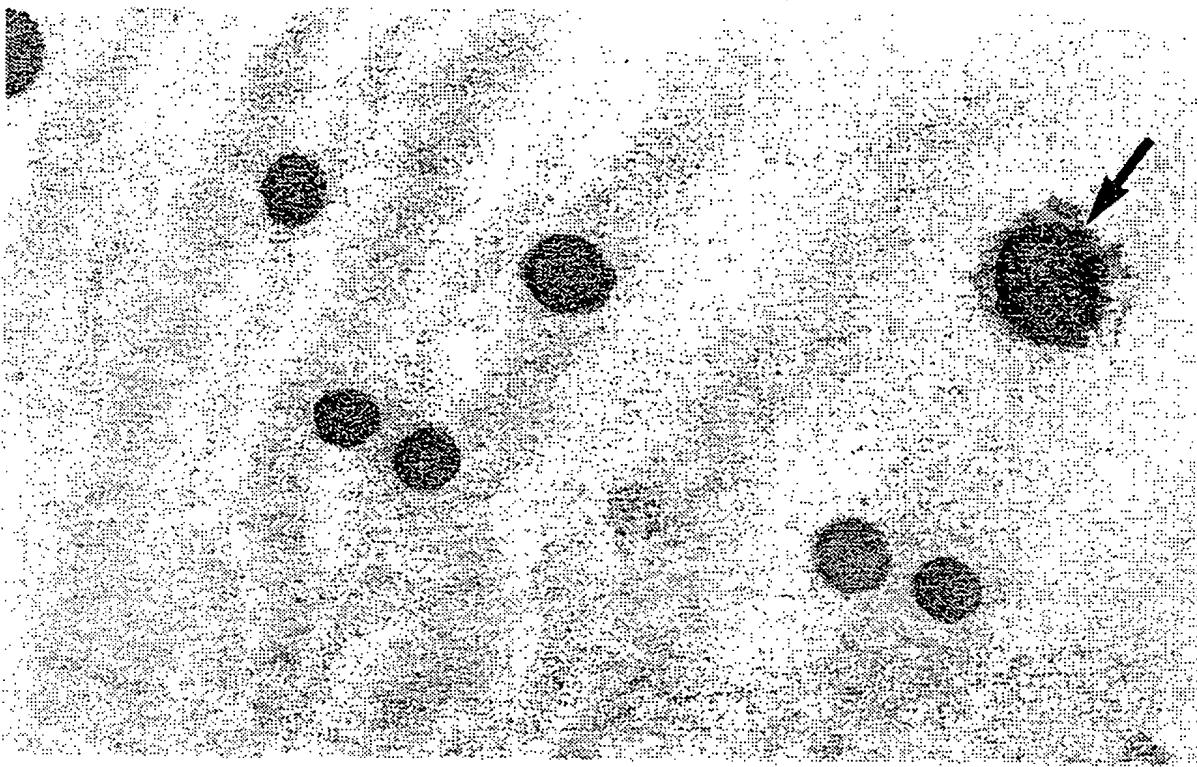
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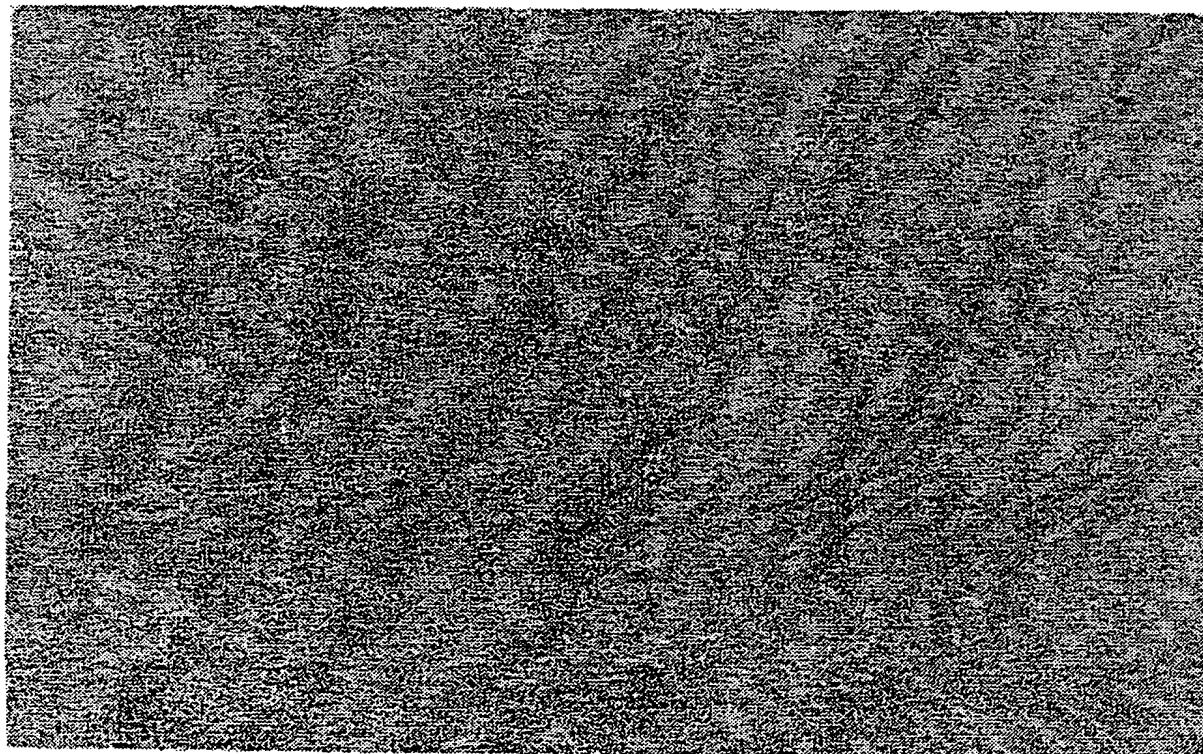
FIGURE 3A

FIGURE 3B   Control
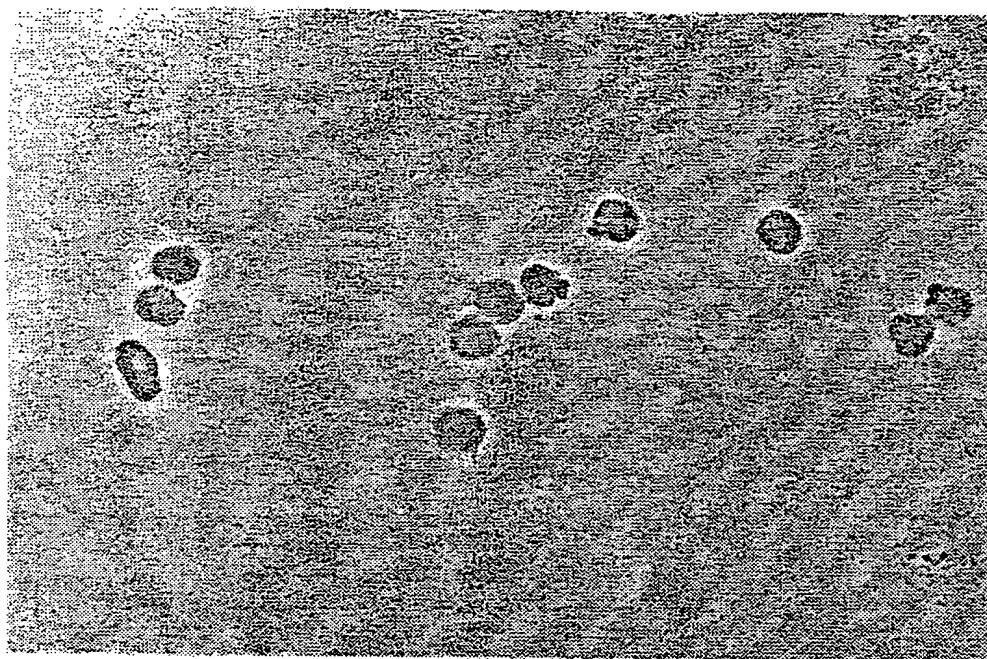
Phase Contrast　　　　　　　　FIGURE 4A

Fluorescent　　　　　　　　FIGURE 4B

pSP72 (Promega)

pCAT wt 760 (Stinski, M. ⌐ T. J. Roehr, J. Virol., 55:431-441, 1985)

Hind III

Hind III
Purify the the fragment containing
hCMV IE enhancer and promoter element

Ligation
Screen for clone in which the enhancer
and promoter element is as following

pZN9

Partial Hind III,
fill in, ligation
Screen for the clone lost
Hind III site 5' to the enhancer
and promoter element

pZN12

pBC12/CMV/IL-2 (Cullen, B. R., Cell 46:973-982, 1986)

Nco I and Hind III
Purify the small fragment

Nco I and Hind III
Purify the large
fragment

Ligation

pZN17

Partial BamH I,
fill in, ligation
Screen for the clone lost BamH I
site at 5'-end of enhancer and
promoter element

pZN13

Hind III and BamH I
Purifiy large fragment

pSV2-cat (Gorman, C. M., et al., Mol. Cel. Biol., 2:1044-1051, 198

Hind III and BamH I
Purifiy Small fragment

Ligation

pZN20

Figure 5

Positions of Restriction Endonucleases sites (unique sites underlined)

```
                    Mae II
                    Aha II
                    Aat II        Mae II
                    SinC II       Mae III            Mae III
                    |  ||         |   |              |
ggcgacogcocagogacoccogocogttgacgtcaatagtgacgtatgttcccatagtaacgccaatagggactttccat 80 (SEQ ID NO: 1)
cogctggogggtcgctggggogggcaactgcagttatcactgcatacaagggtatcattgcggttatccctgaaaggta
     |  ||         |  |           |               |
     26          39  42          57
        29
        29
        30


Mae II
Aha II
Aat II                    Bgl I        Rsa I        Nde I
 ||                       |            |            |
tgacgtcaatgggtggagtatttacggtaaactgcoccacttggcagtacatcaagtgtatcatatgccaagtc-gocooc 160
actgcagttacccaccctcataatgccatttgacgggtgaaccgtcatgtagttcacatagtataogyttcagyogggyy
 ||                       |            |           |
 82                      114          126         141
 82
 83


          Mae II            Mae I             Nla III
          Aha II            Sau96 I           Rsa I
          Aat II            Hae III           Ban I                          Rsa I
          ||                |   |             | | |                          |
tattgacgtcaatgacggtaaatggcccgcctagcattatgcccagtacatgacottacgggagtttcctacttggcagt 240
ataactgcagttactgccatttaccgggcgggatcgtaatacgggtcatgtactggaatgccctcaaaggatgaaccgtca
          ||                |             | | |                              |
          165               184          203                                239
          165               184              206
          166               191              209


                          Sph I
                          Nla III
                          Sty I
                          Sec I
          Mae II          Nco I
          SnaB I          Dsa I    SfaN I        Rsa I                        Ple I
          BsaA I          |  |     |             |                           Hinf I
          ||              || |     |             |                           |
acatctacgtattagtcatcgctattaccatggtgatgcggttttggcagtacaccaatgggcgtggatagcggtttgac 320
tgtagatgcataatcagtagcgataatggtaccactacgccaaaaccgtcatgtggttacccgcacctatcgccaaactg
          ||              || |     |             |                           |
          246             268 275  290                                       318
          246             268
          247             268
                          268
                          269
                          272


                          Mae II
                          Aha II              Nla IV
          BsmA I          Aat II              Ban I
          |               ||                  |
tcacgggggatttccaagtctcaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaa 400
agtgcccctaaaggttcagaggtggggtaactgcagttaccctcaaacaaaacgtggtttttagttgccctgaaaggttt
          |               ||                  |
          337             351                 373
                          351                 373
                          352
```

FIGURE 6A(1)

                                                    Alu I
                                                    Sac I
                                                    BglA I
                    Bga I   Bsp1286 I
                    HinC II   Rsa I Mnl I Ban II

atgtcgtaataaccccgcccccgttgacgcaaatgggcggtaggcgtgtacggtgggaggtctatatagcagagctcgttt 480(SEQ ID NO:
tacagcattattggggcgggggcaactgcgtttacccgccatccgcacatgccaccctccagatatatcgtctcgagcaaa   continued

                    422   447   456   471
                        425   471
                                                       471
                                                       471
                                                       472

                                                    Sau96 I
                 Gsu I   ScrF I   BstU I
                 ScrF I   Mci I   Sau3A I   Sac I
                 EcoR II   Msp I   Mbo I   Sac II
                 BstN I   Fok I   Hpa II Dpn I   BspH II
           Sau3A I   Bga I   Bcn I   Alw I   Dsa I
           Mbo I   Aha II   Mbo II   Ava II   Mnl I
           Dpn I   BamH I   Mnl I   Hbv II   Nla IV   Bgl I

agtgaaccgtcagatcgcctggagacgcatccacgctgttttgaacctcatagaagacaccggyacggatccagcctcc 560
tcacttggcagtctagcggacctctgcggtaggtgcgacaaaactggaggtatcttctgtggcctggctaggtcggagg

           493   502   526   534   543   555
           493   504   534   544   556
           493   504   541   549   559
               498   509   541   549   559
               498   541   549   559
               498   541   549   559
                   499   541   560
                                                       544

     ScrF I
     Mci I
     Msp I
     Hpa II
     Bcn I
    Hae III
   Gdi II
   Eag I
   Eae I
   Fnu4H I   Hinf I   Hae II
   | | | | | BstU I   Hae III

gcggccggggaacggtgcattggaacgcggattocccgtgccaagagtgacgtaagt 616
cgccggcccttgccacgtaacottgcgcctaagggggcacggttctcactgcattca

   | | | | 585   606
   561   589   609
   562
   562
   562
   563
       565
       565
       565
       565
       565

           Restriction Endonucleases site usage

   Aat II   4   BspM I   -   EcoR V   -   Mnl I   3   Rsr II   -
   Acc I   -   BspH I   -   Esp I   -   Msc I   -   Sac I   1
   Afl II   -   BspM II -   Fnu4H I 1   Mse I   -   Sac II   1
   Afl III   -   Bsr I   1   Fok I   1   Msp I   2   Sal I   -
   Aha II   5   BssH II -   Fsp I   -   Nae I   -   Sau3A I   2
   Alu I   1   BstN I   -   Gdi II   1   Nar I   -   Sau96 I   2

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                    FIGURE 6A(2)

Alw I	1	BstE II	-	Gsu I	1	Nci I	2	Sca I	-
AlwN I	-	BstN I	1	Hae I	-	Nco I	1	Scrf I	3
Apa I	-	BstU I	2	Hae II	-	Nde I	1	Sec I	2
ApaL I	-	BstX I	-	Hae III	2	Nhe I	-	SfaN I	1
Ase I	-	BstY I	-	Hga I	2	Nla III	2	Sfi I	-
Asp718	-	Bsu36 I	-	HgiA I	1	Nla IV	2	Sma I	-
Ava I	-	Cfr10 I	-	HgiE II	-	Not I	-	SnaB I	1
Ava II	1	Cla I	-	Hha I	-	Nru I	-	Spe I	-
Avr II	-	Dde I	-	HincC II	2	Nsi I	-	Sph I	-
BamH I	-	Dpn I	2	HinD III	-	Nsp7524 I	-	Spl I	-
Ban I	1	Dra I	-	Hinf I	2	NspB II	1	Ssp I	-
Ban II	1	Dra III	-	HinP I	-	NspH I	-	Stu I	-
Bbe I	-	Drd I	-	Hpa I	-	Pac I	-	Sty I	1
Bbv I	-	Dsa I	2	Hpa II	2	PaeR7 I	-	Taq I	-
Bbv II	1	Eae I	1	Hph I	1	PflM I	-	Tth111 I	-
Bcl I	-	Eag I	1	Kpn I	-	Ple I	1	Tth111 II	-
Bcn I	2	Ear I	-	Mae I	1	Pml I	-	Xba I	-
Bgl I	2	Eco47 III	-	Mae II	7	PpuM I	-	Xca I	-
Bgl II	-	Eco57 I	-	Mae III	3	Pst I	-	Xho I	-
BsaA I	1	EcoN I	-	Mbo I	2	Pvu I	-	Xcm I	-
Bsm I	-	EcoO109 I	-	Mbo II	1	Pvu II	-	Xma I	-
BsmA I	2	EcoR I	-	Nla I	-	Rsa I	5	Xmn I	-
Bsp1286 I	1	EcoR II	1	Mme I	-				

Enzyme	Site		Use	Site position (Fragment length) Fragment order		
Alu I	ag/ct		1	1(471) 1	472(145) 2	
Alw I	ggatc	4/5	1	1(548) 1	549(68) 2	
Ava II	g/gwcc		1	1(543) 1	544(73) 2	
Ban I	g/gyrcc		1	1(372) 1	373(244) 2	
Ban II	grgcy/c		1	1(470) 1	471(146) 2	
Bbv II	gaagac	2/6	1	1(533) 1	534(83) 2	
BsaA I	yac/gtr		1	1(245) 2	246(371) 1	
Bsp1286 I	gdgch/c		1	1(470) 1	471(146) 2	
Bsr I	actgg	1/-1	1	1(202) 2	203(414) 1	
BstN I	cc/wgg		1	1(497) 1	498(119) 2	
Eae I	y/ggccr		1	1(561) 1	562(55) 2	
Eag I	c/ggccg		1	1(561) 1	562(55) 2	
EcoR II	/ccwgg		1	1(497) 1	498(119) 2	
Fnu4H I	gc/ngc		1	1(560) 1	561(56) 2	
Fok I	ggatg	9/13	1	1(508) 1	509(108) 2	
Gdi II	yggccg	-5/-1	1	1(561) 1	562(55) 2	
Gsu I	ctggag	16/14	1	1(498) 1	499(118) 2	
HgiA I	gwgcw/c		1	1(470) 1	471(146) 2	
Hph I	ggtga	8/7	1	1(271) 2	272(345) 1	
Mae I	c/tag		1	1(190) 2	191(426) 1	
Mbo II	gaaga	8/7	1	1(533) 1	534(83) 2	
Nco I	c/catgg		1	1(267) 2	268(349) 1	
Nde I	ca/tatg		1	1(140) 2	141(476) 1	
NspB II	cmg/ckg		1	1(558) 1	559(58) 2	
Ple I	gagtc	4/5	1	1(317) 1	318(299) 2	
Sac I	gagct/c		1	1(470) 1	471(146) 2	
Sac II	ccgc/gg		1	1(558) 1	559(58) 2	
SfaN I	gcatc	5/9	1	1(274) 2	275(342) 1	
SnaB I	tac/gta		1	1(245) 2	246(371) 1	
Sty I	c/cwwgg		1	1(267) 2	268(349) 1	
Bcn I	ccs/gg		2	1(540) 1	541(24) 3	565(52) 2
Bgl I	gccnnnn/nggc		2	1(113) 2	114(441) 1	555(62) 3
BsmA I	gtctc	1/5	2	1(336) 1	337(165) 2	502(115) 3
BstU I	cg/cg		2	1(559) 1	560(25) 3	585(32) 2
Dpn I	ga/tc		2	1(492) 1	493(56) 3	549(68) 2
Dsa I	c/crygg		2	1(267) 2	268(291) 1	559(58) 3
Hae III	gg/cc		2	1(183) 2	184(379) 1	563(54) 3
Hga I	gacgc	5/10	2	1(424) 1	425(79) 3	504(113) 2

FIGURE 6A(3)   **SUBSTITUTE SHEET**

HinC II	gty/rac		2	1(	25) 3	26(	396) 1	422(	199) 2		
Hinf I	g/anto		2	1(	317) 1	318(	271) 2	589(	28) 3		
Hpa II	c/cgg		2	1(	540) 1	541(	24) 3	565(	52) 2		
Mbo I	/gatc		2	1(	492) 1	493(	56) 3	549(	68) 2		
Msp I	c/cgg		2	1(	540) 1	541(	24) 3	565(	52) 2		
Nci I	cc/sgg		2	1(	540) 1	541(	24) 3	565(	52) 2		
Nla III	catg/		2	1(	208) 2	209(	60) 3	269(	348) 1		
Nla IV	ggn/ncc		2	1(	372) 1	373(	170) 2	543(	74) 3		
Sau3A I	/gatc		2	1(	492) 1	493(	56) 3	549(	68) 2		
Sau96 I	g/gncc		2	1(	183) 2	184(	360) 1	544(	73) 3		
Sec I	c/cnngg		2	1(	267) 2	268(	291) 1	559(	58) 3		
Mae III	/gtnac		3	1(	38) 2	39(	18) 3	57(	549) 1	606(	11) 4
Mnl I	ccto	7/7	3	1(	455) 1	456(	70) 2	526(	30) 4	556(	61) 3
ScrF I	cc/ngg		3	1(	497) 1	498(	43) 3	541(	24) 4	565(	52) 2
Aat II	gacgt/c		4	1(	28) 5	29(	53) 4	82(	83) 3	163(	186) 2
				351(	266) 1						
Aha II	gr/cgrc		5	1(	28) 6	29(	53) 5	82(	83) 4	165(	186) 1
				351(	153) 2	504(	113) 3				
Rsa I	gt/ac		5	1(	125) 3	126(	80) 4	206(	33) 6	239(	51) 5
				290(	157) 2	447(	170) 1				
Mae II	a/cgt		7	1(	29) 6	30(	12) 7	42(	41) 5	83(	83) 3
				166(	81) 4	247(	105) 2	352(	257) 1	609(	8) 8

98 sites found

No Sites found for the following Restriction Endonucleases

Acc I	gt/mkac		Dra III	cacnnn/gtg		Nsp7524 I	r/catgy		
Afl II	c/ttaag		Drd I	gacnnnn/nngtc		NspH I	rcatg/y		
Afl III	a/crygt		Ear I	ctcttc	1/4	Pac I	ttaat/taa		
AlwN I	cagnnn/ctg		Eco47 III	agc/gct		PaeR7 I	c/tcgag		
Apa I	gggcc/c		Eco57 I	ctgaag	16/14	PflM I	ccannnn/ntgg		
ApaL I	g/tgcac		EcoN I	cctnn/nnnagg		Pml I	cac/gtg		
Ase I	at/taat		Eco0109 I	rg/gnccy		PpuM I	rg/gwccy		
Asp718	g/gtacc		EcoR I	g/aattc		Pst I	ctgca/g		
Ava I	c/ycgrg		EcoR V	gat/atc		Pvu I	cgat/cg		
Avr II	c/ctagg		Esp I	gc/tnagc		Pvu II	cag/ctg		
BamH I	g/gatcc		Fsp I	tgc/gca		Rsr II	cg/gwccg		
Ehe I	ggcgc/c		Hae I	wgg/ccw		Sal I	g/tcgac		
Ebv I	gcagc	8/12	Hae II	rgcgc/y		Sca I	agt/act		
Bcl I	t/gatca		BglI II	gccnnnnnnggt		Sfi I	ggccnnnn/nggcc		
Bgl II	a/gatct		Nha I	gcg/c		Sma I	ccc/ggg		
Bsm I	gaatgc	1/-1	HinD III	a/agctt		Spe I	a/ctagt		
BspH I	t/catga		HinP I	g/cgc		Sph I	gcatg/c		
BspM I	acctgc	4/8	Hpa I	gtt/aac		Spl I	c/gtacg		
BspM II	t/ccgga		Kpn I	ggtac/c		Ssp I	aat/att		
BssH II	g/cgcgc		Klu I	a/cgcgt		Stu I	agg/cct		
BstB I	tt/cgaa		Mae I	tcgrac	20/18	Taq I	t/cga		
BstE II	g/gtnacc		Mbo I	tgg/cca		Tth111 I	gacn/nngtc		
BstX I	ccannnnn/ntgg		Mse I	t/taa		Tth111 II	caarca	11/9
BstY I	r/gatcy		Nae I	gcc/ggc		Xba I	t/ctaga		
Bsu36 I	cc/tnagg		Nar I	gg/cgcc		Xca I	gta/tac		
Cfr10 I	r/ccggy		Nhe I	g/ctagc		Xho I	c/tcgag		
Cla I	at/cgat		Not I	gc/ggccgc		Xcm I	ccannnnn/nnnntgg	
Dde I	c/tnag		Nru I	tcg/cga		Xma I	c/ccggg		
Dra I	ttt/aaa		Nsi I	atgca/t		Xmn I	gaann/nnttc		

FIGURE 6A(4)

```
••• Aligned sequences:
C1 ( 1f): |>u 1>+++++ ad169hcmv (930 bases)+++++>u 930>|
C2 ( 1f): |>u 1>+++++ townehcmv (616 bases)+++++>u 616>|

••• Alignment of first sequence with all others displayed
••• Key:
    UPPER CASE = aligned non-identical bases
    lower case = unaligned bases
    ---------- = aligned identical bases
    .......... = gap
```

SEQ ID
 NO: 2)   ad169hcmv : AATCAATATTGGCCATTAGCCATATTATTCATTGGTTATATAGCATAAATCAATATTGGC
SEQ ID   townehcmv : ....
 NO: 3)

          ad169hcmv : TATTGGCCATTGCATACGTTGTATCCATATCATAATATGTACATTTATATTGGCTCATGT
          townehcmv : ....

          ad169hcmv : CCAACATTACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACG
          townehcmv : ....

          ad169hcmv : GGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGC
          townehcmv : ....

          ad169hcmv : CCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCC
          townehcmv : ......---.---------G----------G-----------G-------------

          ad169hcmv : ATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACT
          townehcmv : --

          ad169hcmv : GCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAAT
          townehcmv : ---C------------------

          ad169hcmv : GACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACT
          townehcmv : -------------------A-----------------------C----G-----------

          ad169hcmv : TGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTAC
          townehcmv : --

          ad169hcmv : ATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGAC
          townehcmv : -C--

          ad169hcmv : GTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAAC
          townehcmv : --T---

          ad169hcmv : TCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGA
          townehcmv : C--------G--.------

          ad169hcmv : GCTCGTTTAGTGAACCGTCAGATCGCCTGGAGACGCCATCCACGCTGTTTTGACCTCCAT
          townehcmv : --

          ad169hcmv : AGAAGACACCGGGACCGATCCAGCCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATT
          townehcmv : --

          ad169hcmv : CCCCGTGCCAAGAGTGACGTAAGTACCGCCTATAGAGTCTATAGGCCCACCCCCTTGGCT
          townehcmv : ---------------------------.................................

          ad169hcmv : TCTTATGCATGCTATACTGTTTTTGGCTTG
          townehcmv : ....
```

**FIGURE 6B(1)**

# SUBSTITUTE SHEET

```
LOCUS       BS5IEE          930 bp ds-DNA           VRL        15-SEP-1989
DEFINITION  Human cytomegalovirus major immediate-early gene, enhancer.
ACCESSION   X03104
KEYWORDS    major immediate-early gene.
SOURCE      HCMV strain AD169.
  ORGANISM  Human cytomegalovirus
            Viridae; ds-DNA enveloped viruses; Herpesviridae;
            Betaherpesvirinae.
REFERENCE   1 (bases 1 to 930)
  AUTHORS   Boshart,M., Weber,F., Jahn,G., Dorsch-Haesler,K., Fleckenstein,B.
            and Schaffner,W.
  TITLE     A very strong enhancer is located upstream of an immediate early
            gene of human cytomegalovirus
  JOURNAL   Cell 41, 521-530 (1985)
  STANDARD  full automatic
REFERENCE   2 (sites)
  AUTHORS   Zhang,X.-Y., Inamdar,N.M., Supakar,P.C., Wu,K., Ehrlich,M. and
            Ehrlich,K.C.
  TITLE     three MDBP sites in the immediate-early enhancer-promoter region of
            human cytomegalovirus
  JOURNAL   Virology 182, 865-869 (1991)
  STANDARD  full automatic
COMMENT     Draft entry and printed copy of sequence in [1] were kindly
            provided by M.Boshart, 24-OCT-1985.

            The sequence shown is a 930 bp segment of the PstI m-fragment from
            HCMV strain AD169. The enhancer region of the HCMV gene was defined
            by selecting for fragments of HCMV DNA that would restore efficient
            growth of enhancerless SV40.

            A possible splice donor signal is located at positions 856-864.
FEATURES            Location/Qualifiers
     misc_signal    214..620
                    /note="HCMV IE enhancer region"
     mRNA           738..>930
                    /note="HCMV IE mRNA"                      (SEQ ID NO: 2)
BASE COUNT      233 a    228 c    211 g    258 t
ORIGIN      12 bp upstream of BalI site; .750 mu.
        1 aatcaatatt ggccattagc catattattc attggttata tagcataaat caatattggc
       61 tattggccat tgcatacgtt gtatccatat cataatatgt acatttatat tggctcatgt
      121 ccaacattac cgccatgttg acattgatta ttgactagtt attaatagta atcaattacg
      181 gggtcattag ttcatagccc atatatggag ttccgcgtta cataacttac ggtaaatggc
      241 ccgcctggct gaccgcccaa cgaccccgc ccattgacgt caataatgac gtatgttccc
      301 atagtaacgc caatagggac tttccattga cgtcaatggg tggagtattt acggtaaact
      361 gcccacttgg cagtacatca agtgtatcat atgccaagta cgcccctat tgacgtcaat
      421 gacggtaaat ggcccgcctg gcattatgcc cagtacatga ccttatggga ctttcctact
      481 tggcagtaca tctacgtatt agtcatcgct attaccatgg tgatgcggtt ttggcagtac
      541 atcaatgggc gtggatagcg gtttgactca cggggatttc caagtctcca ccccattgac
      601 gtcaatggga gtttgttttg gcaccaaaat caacgggact ttccaaaatg tcgtaacaac
      661 tccgccccat tgacgcaaat gggcggtagg cgtgtacggt gggaggtcta tataagcaga
      721 gctcgtttag tgaaccgtca gatcgcctgg agacgccatc cacgctgttt tgacctccat
      781 agaagacacc gggaccgatc cagcctccgc ggccgggaac ggtgcattgg aacgcggatt
      841 ccccgtgcca agagtgacgt aagtaccgcc tatagagtct ataggcccac cccttggct
      901 tcttatgcat gctatactgt ttttggcttg
```

FIGURE 6B(2)

SUBSTITUTE SHEET

```
LOCUS        BS5MIE1          616 bp ds-DNA          VRL      15-SEP-1989
DEFINITION   Human cytomegalovirus (Towne) major immediate-early (IE) gene, exon
             1.
ACCESSION    K01484 K01090
KEYWORDS     major immediate-early gene.
SEGMENT      1 of 4
SOURCE       Human cytomegalovirus (strain Towne) passed in primary human
             foreskin fibroblasts, DNA [1], clone pXEP22 [2].
  ORGANISM   Human cytomegalovirus
             Viridae; ds-DNA enveloped viruses; Herpesviridae;
             Betaherpesvirinae.
REFERENCE    1  (bases 460 to 616)
  AUTHORS    Stenberg,R.M., Thomsen,D.R. and Stinski,M.F.
  TITLE      Structural analysis of the major immediate early gene of human
             cytomegalovirus
  JOURNAL    J. Virol. 49, 190-199 (1984)
  STANDARD   full automatic
REFERENCE    2  (bases 1 to 490)
  AUTHORS    Thomsen,D.R., Stenberg,R.M., Goins,W.F. and Stinski,M.F.
  TITLE      Promoter-regulatory region of the major immediate early gene of
             human cytomegalovirus
  JOURNAL    Proc. Natl. Acad. Sci. U.S.A. 81, 659-663 (1984)
  STANDARD   full automatic
COMMENT      IE region 1 gene is also known as the major IE gene.

             Cytomegalovirus immediate-early gene expression is dominated in
             vivo by the expression of a single gene. At least three promoters
             influence transcription of the virus after infection. When a
             complete set of promoter-regulatory regions were present (IE
             regions 1, 2 and 3 or IE region 1 and an adenovirus major late
             promoter), transcription was qualitatively higher from IE region 1.

             Based on these data, [1] proposes that the upstream sequence of the
             IE region 1 gene competes more efficiently for RNA polymerase II or
             other host cell proteins necessary for in vitro transcription.

             Consensus CAAT and TATA boxes were found at positions 429-433 and
             462-467, a polyadenylation signal was found at positions 2198-2203.

             Fourteen direct repeats were found in the promoter-regulatory
             region (four 16-bp repeats, four 18-bp repeats, four 19-bp repeats
             and two 21-bp repeats).

             Draft entry and clean copy sequences [1],[2] kindly provided by
             P.R. Witte and M.F. Stinski (10-FEB-1986).
FEATURES              Location/Qualifiers
     prim_transcript  490..>616
                      /note="major IE mRNA"
     intron           611..>616                          (SEQ ID NO: 3)
                      /note="major IE mRNA intron A"
BASE COUNT      144 a     165 c      162 g       145 t
ORIGIN       28 bp upstream of BincII site; 0.752 map units.
       1 ggcgaccgcc cagcgacccc cgcccgttga cgtcaatagt gacgtatgtt cccatagtaa
      61 cgccaatagg gactttccat tgacgtcaat gggtggagta tttacggtaa actgcccact
     121 tggcagtaca tcaagtgtat catatgccaa gtccgccccc tattgacgtc aatgacggta
     181 aatggcccgc ctagcattat gcccagtaca tgaccttacg ggagtttcct acttggcagt
     241 acatctacgt attagtcatc gctattacca tggtgatgcg gttttggcag tacaccaatg
     301 ggcgtggata gcggtttgac tcacggggat ttccaagtct ccaccccatt gacgtcaatg
     361 ggagtttgtt ttggcaccaa aatcaacggg actttccaaa atgtcgtaat aaccccgccc
     421 cgttgacgca aatgggcggt aggcgtgtac ggtgggaggt ctatatagca gagctcgttt
     481 agtgaaccgt cagatcgcct ggagacgcca tccacgctgt tttgacctcc atagaagaca
     541 ccgggaccga tccagcctcc gcggccggga acggtgcatt ggaacgcgga ttccccgtgc
     601 caagagtgac gtaagt
```

SUBSTITUTE SHEET

FIGURE 6B(3)

Positions of Restriction Endonucleases sites (unique sites underlined)

```
    Rae III
    Mao I
    Sae I                                          Rae III
    Xae I                                          Mac I
                                                   Rae I
    Ssp I                           Ssp I          Rae I              Mae II
     |                               |              ||                 |
aatcaatattggccattagccatattattcattggttatatagcataaatcaatattggctattggccattgcataogtt  80 (SEQ ID NO: 4)
ttagttataacoggtaatcggtataataagtaaccaatatatogtatttagttataacogataacoggtaacogtatgcaa
 |     ||                                            |      ||                |
 5    10                                            52     64               76
      10                                                   64
      10                                                   64
      11                                                   65
```

```
                            Mae I                  HinC II                Mae I
            Rsa I           Nla III                Nla III                Spe I
             |               |   |                  |   |                  ||
gtatocatatcataatatgtacatttatattggctcatgtccaacattacogcoatgttgacattgattattgactagtt  160
catagytatagtattatacatgtaaatataacogagtacaggttgtaatggcggtacaactgtaactaataactgatcaa
             |               |   |                  |   |                  ||
            99              116 120                134 137                154 155
```

```
Mae I                                       Mae III                      Bgl I
Ase I                                        BstU I                      Sau96 I
 ||                                          |   |                        Rae III
attaatagtaatcaattacggggtcattagttcatagccatatatggagttcogcgttacataacttacggtaaatggc  240
taattatcattagttaatgcoccagtaatcaagtatcgggtatataoctcaaggcgcaatgtattgaatgocatttaocg
 ||                                          |   |                        ||
161                                         214 217                      238
162                                             217                      238
                                                                         239
```

```
ScrF I                  Mae II
EcoR II                 Aha II
BstN I                  Aat II           Mae II              Mae III
 |                       ||               |                  |
cogcctggctgacogcccaacgacocoogcccattgacgtcaataatgacgtatgttcocatagtaacgocaataggggac  320
ggcggacogactggcgggttgctgggggcgggtaactgcagttattactgcatacaaggctatcattgcggttatocctg
 |                       ||               |                  |
244                     276             289                304
244                     276
244                     277
```

```
Mae II
Aha II
Aat II                  Bgl I           Rsa I              Nde I        Rsa I
 ||                      |               |                  |            |
tttocattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgocaagta  400
aaaggtaactgcagttacocacctcataaatgocatttgacggtgaacogtcatgtagttcacatagtatacggttcat
 ||                      |               |                  |            |
329                     361             373                388          398
329
330
```

```
                                        ScrF I
                                        EcoR II
                                        BstN I
        Mae II                  Bgl I                        Nla III
        Aha II                  Sau96 I             Rsa I
        Aat II                  Rae III             Bar I
         ||                      ||   |              |   |
cgcccoctattgacgtcaatgacggtaaatggccocgoctggcattatgoccagtacatgaocttatgggactttootact  480
gcggggggataactgcagttactgocatttaocgggcggacogtaataogggtcatgtactggaataocotgaaagggatga
         ||                      ||   |              |   |
        412                     431   |             450  |
        412                     431                     453
        413                     432                     456
                                437
                                437
                                437
```

FIGURE 6C(1)   **SUBSTITUTE SHEET**

```
                                    Sph I
                                    Xho III
                                    Sty I
                           Mae II    Sec I
                           SnaB I    Nco I
                  Rsa I    BsaA I    Dsa I    SfaN I        Rsa I         (SEQ ID NO: 4)
                    |        |         ||       |            |             continued
tggcagtacatctacgtattagtcatcgctattaccatggtgatgcggtttttggcagtacatcaatgggcgtggatagcg  560
accgtcatgtagatgcataatcagtagcgataatggtaccactacgccaaaaccgtcatgtagttacccgcacctatcgc
                    |        ||                ||    | |               |
                  486      493              515   522             537
                           493              515
                           494              515
                                            515
                                            516
                                            519
                                            Mae II
                                  Pla I     Aha II                Xha IV
                                  Hinf I    Aat II                Ban I
                  |        |         ||       |            |            |
gtttgactcacggggatttccaagtctcacccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggact  640
caaactgagtgcccctaaaggttcagagtggggtaactgcagttacccctcaaacaaaaccgtggtttttagttgccctga
                  |        |         ||                             |
                565      584       598                           620
                565                598                           620
                                   599
```

```
                                                                      Alu I
                                                                      Sac I
                                                                      Bgl I
                                                                      Bsp1286 I
                  Mae III          Hga I              Rsa I    Nhl I   Ban II
                    |                |                  |        |       ||
ttccaaaatgtcgtaacaactccgcccccattgacgcaaatgggcggtaggcgtgtacggtgggaggtctatataagcaga  720
aaggttttacagcattgttgaggcggggtaactgcgtttacccgccatccgcacatgccacccctccagatatattcgtct
                    |                |                  |        |       ||
                  653              672                694      703     719
                                                                      719
                                                                      719
                                                                      719
                                                                      720
```

```
                                                                    Sau96 I
                                                                    ScrF I
                                              Gsu I                  Hci I    Sau3A I
                                              ScrF I                 Hap I    Mbo I
                                              EcoR II                Rpa II   Dpn I
                                              BstN I    Fok I        Bcn I    Alw I
                           Sau3A I    Hga I                    Mbo II Ava II
                           Mbo I      Aha II              Nhl I Bbv II Xha IV
                           Dpn I      BsmA I               |     |     |||
gctcgtttagtgaaccgtcagatcgcctggagacgccatccacgctgtttttgacctccatagaagacaccgggacggatc  800
cgagcaaatcacttggcagtctagcggacctctgcggtaggtgcgacaaaactggaggtatcttctgtggccctggctag
                           |     ||    |    |                |     |     |  || |
                         741   750                         774   782   791
                         741   752                               782   792
                         741   752                                     789   797
                              746                                       789   797
                              746      757                              789   797
                              746                                       789   797
                              747                                       789
                                                                              792
```

FIGURE 6C(2)

SUBSTITUTE SHEET

```
                  Scrf I
                  Mci I
                  Msp I
                  Hpa II
                  Bcn I
              Gdi II
              Eag I
              Fnu4H I
             BstU I
            Sec I
            Sac II
            MspB II
            Dsa I
       Mnl I    Hae III                Hinf I              Hae II              Ple I
       Bgl I  Hae I                    BstU I         Hae III   Rsa I         Hinf I      (SEQ ID NO: 4)
       | |  ||||| |                      |   |          |   |     |            |          continued
cagcctccgcggccgggaacggtgcattggaacgcggattccccgtgccaagagtgacgtaagtaccgcctatagagtct  880
gtcggaggcgccggcccttgccacgtaaccttgcgcctaagggggcacggttctcactgcattcatggcggatatctcaga
       | |  ||||| |                      |   |          |   |     |            |
      803   810                         833           854    863           875
        804   811                         837           857                 875
          807
          807
          807
          807
           808
             809
              810
              810
                 813
                 813
                 813
                 813
                 813
```

```
                          Nla III
                          Sph I
                          NspH I
            BstX I         Nsp7524 I
        Sau96 I  Sty I     Nsi I
        Hae III  Sec I      | ||
          |   |    |        | ||
ataggcccaccccccttggcttcttatgcatgctatactgttttttggcttg  930
tatccgggtgggggggaaccgaagaataacgtacgatatgacaaaaaaccgaac
          |   |    |        | ||
         884    893       905
         884    893        907
           887              907
                           907
                            908
```

Restriction Endonucleases site usage

Aat II	4	BspH I	–	EcoR V	–	Nal I	3	Bsr II	–
Aco I	–	BspM I	–	Esp I	–	Mae I	2	Sac I	1
Afl II	–	BspM II	–	Fnu4H I	1	Mse I	1	Sac II	1
Afl III	–	Bsr I	1	Fok I	1	Msp I	2	Sal I	–
Aha II	5	BssH II	–	Fsp I	1	Mae I	–	Sau3A I	2
Alu I	1	BstB I	–	Gdi II	1	Nar I	–	Sau96 I	4
Alw I	1	BstE II	–	Gsu I	1	Ncl I	2	Sca I	–
AlwN I	–	BstN I	3	Hae I	2	Nco I	1	Scrf I	5
Apa I	–	BstU I	3	Hae II	–	Nde I	1	Sec I	3
ApaL I	–	BstX I	1	Hae III	6	Nhe I	–	SfaN I	1
Ase I	1	BstY I	–	Hga I	2	Nla III	5	Sfi I	–
Asp718	–	Bsu36 I	–	HgiA I	1	Nla IV	2	Sma I	–
Ava I	–	Cfr10 I	–	HgiE II	–	Not I	–	SnaB I	1
Ava II	1	Cla I	–	Nha I	–	Nru I	–	Spe I	1
Avr II	–	Dde I	–	HinC II	1	Nsi I	1	Sph I	1
BamH I	–	Dpn I	2	HinD III	–	Nsp7524 I	1	Spl I	–
Ban I	1	Dra I	–	Hinf I	3	NspB II	1	Ssp I	2
Ban II	1			Nla I	–	NspH I	1	Stu I	–
```
                FIGURE 6C(3)  SUBSTITUTE SHEET
```

Bbe I	-	Drd I	-	Spe I	-	Pac I	-	Sty I	2
Bbv I	-	Dsa I	2	Spe II	2	PaeR7 I	-	Taq I	-
Bbv II	1	Eae I	3	Sph I	1	Pf1M I	-	Tth111 I	-
Bcl I	-	Eag I	1	Kpn I	-	Ple I	2	Tth111 II	-
Bcn I	2	Ear I	-	Mae I	1	Pml I	-	Xba I	-
Bgl I	4	Eco47 III	-	Mae II	8	PpuM I	-	Xca I	-
Bgl II	-	Eco57 I	-	Mae III	4	Pst I	-	Xho I	-
BsaA I	1	EcoN I	-	Mbo I	2	Pvu I	-	Xcm I	-
Bsm I	-	EcoO109 I	-	Mbo II	1	Pvu II	-	Xma I	-
BsmA I	2	EcoR I	-	Alu I	-	Rsa I	8	Xmn I	-
Bsp1286 I	1	EcoR II	3	Mme I	1				

Enzyme	Site	Use	Site position (Fragment length) Fragment order		
Alu I	ag/ct	1	1(719) 1	720(211) 2	
Alw I	ggatc	4/5	1	1(796) 1	797(134) 2
Ase I	at/taat	1	1(160) 2	161(770) 1	
Ava II	g/gwcc	1	1(791) 1	792(139) 2	
Ban I	g/gyrcc	1	1(619) 1	620(311) 2	
Ban II	grgcy/c	1	1(718) 1	719(212) 2	
Bbv II	gaagac	2/6	1	1(781) 1	782(149) 2
BsaA I	yac/gtr	1	1(492) 1	493(438) 2	
Bsp1286 I	gdgch/c	1	1(718) 1	719(212) 2	
Bsr I	actgg	1/-1	1	1(449) 2	450(481) 1
BstX I	ccannnnn/ntgg	1	1(886) 1	887(44) 2	
Eag I	c/ggccg	1	1(809) 1	810(121) 2	
Fnu4H I	gc/ngc	1	1(808) 1	809(122) 2	
Fok I	ggatg	9/13	1	1(756) 1	757(174) 2
Gdi II	yggccg	-5/-1	1	1(809) 1	810(121) 2
Gsu I	ctggag	16/14	1	1(746) 1	747(184) 2
HgiA I	gwgcw/c	1	1(718) 1	719(212) 2	
HinC II	gty/rac	1	1(136) 2	137(794) 1	
Hph I	ggtga	8/7	1	1(518) 1	519(412) 2
Mae I	c/tag	1	1(154) 2	155(776) 1	
Mbo II	gaaga	8/7	1	1(781) 1	782(149) 2
Mme I	tccrac	20/18	1	1(119) 2	120(811) 1
Mse I	t/taa	1	1(161) 2	162(769) 1	
Mco I	c/catgg	1	1(514) 1	515(416) 2	
Nde I	ca/tatg	1	1(387) 2	388(543) 1	
Nai I	atgca/t	1	1(904) 1	905(26) 2	
Msp7524 I	r/catgy	1	1(906) 1	907(24) 2	
MspB II	cmg/ckg	1	1(806) 1	807(124) 2	
MspR I	rcatg/y	1	1(906) 1	907(24) 2	
Sac I	gagct/c	1	1(718) 1	719(212) 2	
Sac II	ccgc/gg	1	1(806) 1	807(124) 2	
SfaN I	gcatc	5/9	1	1(521) 1	522(409) 2
SnaB I	tac/gta	1	1(492) 1	493(438) 2	
Spe I	a/ctagt	1	1(153) 2	154(777) 1	
Sph I	gcatg/c	1	1(906) 1	907(24) 2	

Enzyme	Site	Use					
Bcn I	ccs/gg	2	1(788) 1	789(24) 3	813(118) 2	
BsmA I	gtctc	1/5	2	1(583) 1	584(166) 3	750(181) 2
Dpn I	ga/tc	2	1(740) 1	741(56) 3	797(134) 2	
Dsa I	c/crygg	2	1(514) 1	515(292) 2	807(124) 3	
Eae I	wgg/ccw	2	1(9) 3	10(54) 2	64(867) 1	
Eca I	gacgc	5/10	2	1(671) 1	672(80) 3	752(179) 2
Spe II	c/cgg	2	1(788) 1	789(24) 3	813(118) 2	
Mbo I	/gatc	2	1(740) 1	741(56) 3	797(134) 2	
Mec I	tgg/cca	2	1(9) 3	10(54) 2	64(867) 1	
Msp I	c/cgg	2	1(788) 1	789(24) 3	813(118) 2	
Nci I	cc/sgg	2	1(788) 1	789(24) 3	813(118) 2	
Nla IV	ggn/ncc	2	1(619) 1	620(171) 2	791(140) 3	
Ple I	gagtc	4/5	2	1(564) 1	565(310) 2	875(56) 3
Sau3A I	/gatc	2	1(740) 1	741(56) 3	797(134) 2	
Ssp I	aat/att	2	1(4) 3	5(47) 2	52(879) 1	
Sty I	c/cwgg	2	1(514) 1	515(378) 2	893(38) 3	

FIGURE 6C(4) **SUBSTITUTE SHEET**

BstN I	cc/wgg	3	1(	243)	2	244(	193)	3	437(	309)	1	746(	195)	4
BstU I	cg/cg	3	1(	213)	2	214(	594)	1	808(	25)	4	833(	98)	3
Eae I	y/ggccr	3	1(	9)	4	10(	54)	3	64(	746)	1	810(	121)	2
EcoR II	/ccwgg	3	1(	243)	2	244(	193)	3	437(	309)	1	746(	195)	4
Hinf I	g/antc	3	1(	564)	1	565(	272)	2	837(	38)	4	875(	56)	3
Mnl I	cctc 7/7	3	1(	702)	1	703(	71)	3	774(	30)	4	804(	127)	2
Sec I	c/cnngg	3	1(	514)	1	515(	292)	2	807(	86)	3	893(	38)	4
Aat II	gacgt/c	4	1(	275)	2	276(	53)	5	329(	83)	4	412(	186)	3
			598(| 333)	1									
Bgl I	gccnnnn/nggc	4	1(	238)	2	239(	122)	4	361(	71)	5	432(	371)	1
			803(| 128)	3									
Mae III	/gtnac	4	1(	216)	2	217(	87)	4	304(	349)	1	653(	201)	3
			854(| 77)	5									
Sau96 I	g/gncc	4	1(	237)	2	238(	193)	3	431(	361)	1	792(	92)	4
			884(| 47)	5									
Aha II	gr/cgyc	5	1(	275)	1	276(	53)	6	329(	83)	5	412(	186)	2
			598(| 154)	4	752(| 179)	3						
Nla III	catg/	5	1(	115)	3	116(	18)	6	134(	322)	2	456(	60)	4
			516(| 392)	1	908(| 23)	5						
ScrF I	cc/ngg	5	1(	243)	2	244(	193)	3	437(	309)	1	746(	43)	5
			789(| 24)	6	813(| 118)	4						
Hae III	gg/cc	6	1(	10)	7	11(	54)	5	65(	173)	3	238(	193)	2
			431(| 380)	1	811(| 73)	4	884(| 47)	6				
Mae II	a/cgt	8	1(	75)	6	76(	201)	2	277(	12)	9	289(	41)	8
			330(	83)	4	413(	81)	5	494(	105)	3	599(	258)	1
			857(| 74)	7									
Rsa I	gt/ac	8	1(	98)	4	99(	274)	1	373(	25)	9	398(	55)	6
			453(	33)	8	486(	51)	7	537(	157)	3	694(	169)	2
			863(| 68)	5									

141 sites found

No Sites found for the following Restriction Endonucleases

Acc I	gt/mkac		Dde I	c/tnag		Nru I	tcg/cga
Afl II	c/ttaag		Dra I	ttt/aaa		Pac I	ttaat/taa
Afl III	a/crygt		Dra III	cacnnn/gtg		PaeR7 I	c/tcgag
Alw I	cagnnn/ctg		Drd I	gacnnnn/nngtc		PflM I	ccannnn/ntgg
Apa I	gggcc/c		Ear I	ctcttc 1/4		Pml I	cac/gtg
ApaL I	g/tgcac		Eco47 III	agc/gct		PpuM I	rg/gwccy
Asp718	g/gtacc		Eco57 I	ctgaag 16/14		Pst I	ctgca/g
Ava I	c/ycgrg		EcoN I	cctnn/nnnagg		Pvu I	cgat/cg
Avr II	c/ctagg		Eco0109 I	rg/gnccy		Pvu II	cag/ctg
BamH I	g/gatcc		EcoR I	g/aattc		Rsr II	cg/gwccg
Bbe I	ggcgc/c		EcoR V	gat/atc		Sal I	g/tcgac
Bbv I	gcagc 8/12		Esp I	gc/tnagc		Sca I	agt/act
Bcl I	t/gatca		Fsp I	tgc/gca		Sfi I	ggccnnnn/nggcc
Bgl II	a/gatct		Hae II	rgcgc/y		Sma I	ccc/ggg
Ban I	gaatgc 1/-1		BglII II	accnnmnnnggt		Spl I	c/gtacg
BspH I	t/catga		Nha I	gcg/c		Stu I	agg/cct
BspM I	acctgc 4/8		HinD III	a/agctt		Taq I	t/cga
BspM II	t/ccgga		HinP I	g/cgc		Tth111 I	gacn/nngtc
BssH II	g/cgcgc		Hpa I	gtt/aac		Tth111 II	caarca 11/9
BstB I	tt/cgaa		Kpn I	ggtac/c		Xba I	t/ctaga
BstE II	g/gtnacc		Nla I	a/cgcgt		Xca I	gta/tac
BstY I	r/gatcy		Mae I	gcc/ggc		Xho I	c/tcgag
Bsu36 I	cc/tnagg		Nar I	gg/cgcc		Xcm I	ccannnn/nnnntgg
Cfr10 I	r/ccggy		Nhe I	g/ctagc		Xma I	c/ccggg
Cla I	at/cgat		Not I	gc/ggccgc		Xmn I	gaann/nnttc

INTERNALIZATION OF LIPID DNA COMPLEXES IN CV-1 CELLS

FIGURE 7

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FIGURE 8A

FIGURE 8B

FIGURE 8C   Control

Figure 9

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pZN13   pSP72   pBC/CMV/IL-2 (Cullen. B. R.. Cell 46:973-982. 1986)

Bgl II. fill in. then Xba I

Gel purify fragment containing
hCMV enhancer-promoter (760bp)

Pvu II+Xba I

Gel purify the large fragment

Partial digestion with Nde I. fill in. gel purify
the band at 4570bp.
Ligation. screen clone deleted a fragment
from Nde I(1755) to Nde I(1917)

Ligation

pZN15

Partial digestion with EcoR I. fill in. gel purify
the band cut once by EcoR I.
Ligation. screen for clone lost EcoR I(1631)

pZN22

PpuM I. fill in. then EcoR I

Gel purify the fragment containing
intron and polyA site (665bp)

Sma I+EcoR I
Gel purify the large fragment

Ligation

pSV2-cat (Gorman. C. M.. et al..
Mol. Cel. Biol., 2:1044-1051. 1982)

pSV2-cat (Gorman. C. M.. et al..
Mol. Cel. Biol., 2:1044-1051. 1982)

Ban I. fill in.
then Hind III

Gel purify the CAT
containing
fragment (716bp)

Acc I. fill in
then Hind III

Gel purify the
large fragment

Sma I

Gel purify the fragment

Ban I+Hind III. fill in

Gel purify the CAT
containing
fragment (716bp)

Sites removed

Ligation

Ligation
Screen for clone containing the CAT
gene in sense orientation

Sites removed   Sites removed

Figure 10 A

pZN29

pBC12/CMV/IL-2
(Cullen, B. R., Cell 46:973-982, 1986)

Hind III+BamH I

Hind III+BamH I

Gel purify large fragment

Gel purify fragment containing
IL-2 Gene (684bp)

Ligation

Figure 10B

pBQ4.7 (CFTR cDNA clone, from Dr. Francis Collins)   pZN29

Pst I, fill in, gel purify CFTR cDNA insert (~4.7kb)   Sma I

Ligation
Screen for clone containing insert
in the sense orietation

Figure 11
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FIGURE 12A   50X

FIGURE 12B   Control 50X

SUBSTITUTE SHEET

FIGURE 12C   100X

FIGURE 12D   Control 100X

FIGURE 12E   250X

pZN26   pSV2-cat (Gorman, C. M., et al.,
Mol. Cel. Biol., 2:1044-1051, 1982)

EcoR V   Ase I+BamH I, fill in
Gel purify insert containing polyA site (~330bp)

Ligation
Screen for clone containing insert
in an orientation as following

Nde I   Xho I
Aat II
Ssp I
SP6
hCMV(Towne)
Xmn I
hCMV(AD169)
Sca I
Pvu I
Amp   **pZN35**   PolyA
Fsp I
Bgl I
T7
ori
Hpa I

Hind III
Sph I
Pst I
Sal I
Acc I
Xba I
BamH I
Sma I
Kpn I
Sac I
EcoR I
Cla I

Bgl II

pSV2-cat (Gorman, C. M., et al.,
Mol. Cel. Biol., 2:1044-1051, 1982)

Acc I, fill in,
then Hind III
Gel purify the large fragment

Ban I, fill in, then Hind III
Gel purify inser containing
CAT gene (~720bp)

Ligation
Screen for clone containing CAT gene
in sense orientation

Nde I   Xho I
Aat II
Ssp I
SP6
hCMV(Towne)
Xmn I
hCMV(AD169)
Sca I
Hind III
Pvu I
Fsp I
Amp
Bgl I   **pZN51**   CAT
ori
PolyA
T7
Xba I
BamH I
Sma I
Kpn I
Sac I
EcoR I
Cla I
Bgl II
Hpa I

Figure 13

Figure 14

SUBSTITUTE SHEET

Figure 14A

Figure 14B

Figure 14c

FIGURE 15
SUBSTITUTE SHEET

FIGURE 16A

FIGURE 16B

FIGURE 16C   Control

FIGURE 16D

FIGURE 16E

pBQ4.7 (CFTR cDNA clone, from Dr. Francis Collins)   pZN76

Xma I and Sal I,   Xma I and Sal I
gel purify the large fragment

Ligation

Figure 18

SUBSTITUTE SHEET

FIGURE 19A

CAT EXPRESSION WITH pZN27 COMPLEXED TO A LIPID CARRIER

CAT Gene Product
[14C acetyl-Chloramphenicols]

Unincorporated Label
[14C Chloramphenicol]

Origin

Pancreas
Muscle
Ovary
Uterus/Oviduct
Caecum
Small Intestine
Stomach
Spleen
Liver
Heart
Trachea
Thymus
Lung
Muscle
Kidney
Liver
Lung
Control

FIGURE 19B

SUBSTITUTE SHEET

CAT EXPRESSION AFTER INJECTION OF PLASMID INTO MOUSE BRAIN RIGHT VENTRICLES

CAT Gene Product [14C acetyl-Chloramphenicols]

Unincorporated Label [14C Chloramphenicol]

Origin Ventricle

(plasmid:lipid, μg:nmoles)

1:0   1:1   1:3   1:4   0:6

FIGURE 20

SUBSTITUTE SHEET

CAT EXPRESSION AFTER TRANSFECTION
OF HUMAN LUNG CANCER CELL LINES

FIGURE 21

IMMUNOREACTIVE GM-CSF

(Expressed relative to pre-inoculation value)

FIGURE 22

IMMUNOREACTIVE GM-CSF
(Expressed relative to pre-inoculation value)

FIGURE 23